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A study of the role of genetic factors in the classification of distinct multiple sclerosis clinical phenotypes

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A Study of the Role of Genetic Factors in the Classification of Distinct Multiple Sclerosis Clinical Phenotypes

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Summary

The following thesis tests the working hypothesis that the distinct clinical manifestations of multiple sclerosis (MS) may be underpinned by phenotype specific differences in gene expression profiles in T-lymphocytes.
The first objective of the thesis was to examine *in silico* gene expression libraries derived from normal brain and MS brain, in order to identify differential gene expression in MS brain lesions. From the cohort of genes identified, genes whose function was likely to be implicated in T-lymphocyte activation were examined in T-lymphocyte preparations from patients with each of the MS phenotypes and healthy controls.

Subsequently, global T-lymphocyte gene expression profiles were compared across MS clinical phenotypes and healthy controls using microarray technology and classification of the major changes in gene expression were made. The expression of a cohort of genes potentially implicated in T-lymphocyte activation were confirmed by real-time RT-PCR and examined in myelin basic protein (MBP)/interleukin-2 stimulated control T-lymphocyte cultures.
Lastly, a single nucleotide polymorphism study was carried out in an Irish family with four MS affected siblings, one with secondary progressive MS (SP MS) remainder present with relapsing remitting MS (RR MS).

Overall, the work presented herein demonstrates the utility of a multi-faceted approach to examining gene expression profiles in MS. The data obtained from the *in silico* studies are robust enough to provide targets which were able to be

validated in clinical samples. Elsewhere, microarray comparisons provided interesting evidence pointing to the existence of MS phenotype specific T-lymphocyte activation profiles, at least in terms of the transcriptomic response. The family study in conclusion was underpowered in terms of identifying disease loci, but did however demonstrate that there are regions of the genome clearly shared by the affected siblings (281 words).

Declaration of Author

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree PhD is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Student Number **04236181**

Date **6/11/2008**

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In silico **transcriptome analysis identifies genes whose expression is altered in Multiple Sclerosis.**

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***In silico* profiling identifies novel patterns of gene expression in multiple sclerosis.**

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High-density scan for genetic linkage to Multiple Sclerosis (MS) in a multiply affected family

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To my parents, sisters, my brother in law, my niece and three nephews-
thank you for all your support and encouragement.

Abbreviations

Ab Antibody

α- B-C α-B crystalline

APO-E Apolipoprotein E

ACTH Adrenocorticotophic hormone

APC Antigen- presenting cell

APL Altered peptide ligand

BBB Blood- brain barrier

CCR3, CCR5 chemokine receptor

C3d Complement 3 d

CIS Clinically isolated syndrome

CM Complete medium

CNS Central nervous system

CO2 Carbon dioxide

CSF Cerebrospinal fluid

CT Computed tomography

CTLA Cytotoxic T lymphocyte- associated antigen

CXCR3 chemokine receptor, G protein- coupled receptor 3

DEPC is deionized, diethylpyrocarbonate (DEPC)-treated Water

DNA Deoxyribonucleic acid

EAE Experimental autoimmune (allergic) encephalomyelitis

EBV Ebstein-Barr virus

EDSS Expanded disability status score

EDTA ethylenediaminetetraacetic acid- a chelating agent

ELISA Enzyme- linked immunosorbent assay

EP Evoked potentials

EtBr Ethidium bromide

FLAIR Fluid- attenuated inversion recovery

FS Functional score

GFAP Glial fibrillary acidic protein

GRE Gradient recalled echo

H^+ Hydrogen ion

HBSS Gibco- Hank's balanced salts

HHV-6 Human herpesvirus type 6

HLA Human leukocyte antigen

H2SO4 Sulphuric acid

HSP Heat shock protein

IFN Interferon

ICAM Intercellular adhesion molecule

IgA Immunoglobulin A

IgG Immunoglobulin G

IgM Immunoglobulin M

IL Interleukin

INO Internuclear opthalmoplegia

IR Inversion recovery

LFA-1 Lymphocyte function associated antigen 1

LFB Luxol fast blue

LT Leukotrien P38 Lymphotoxin

MAG Myelin- associated protein

MBP Myelin basic protein

MHC Major histocompatibility complex

MMP-9 Matrix metaloproteinase 9

MOBP Myelin- associated oligodendrocytic basic protein

MOG Myelin oligodendrocyte glycoprotein

MRI Magnatic resonance imaging

mRNA Messanger RNA

MRS Magnetic resonance spectroscopy

MS Multiple sclerosis

NK Natural killer

NMR Nuclear magnetic resonance

Nogo Neurite inhibitory outgrowth protein

OCB Oligoclonal bands

Omgp Oligodendrocyte myelin glycoprotein

ON Optic neuritis

OSP Oligodendrocyte- spcific glycoprotein

PAS Periodic schiff

PBMC Polymorphnuclear cells

PBS Phosphate buffer salt

PLP Proteolipid protein

RANTES: CCL5, a chemokine

RAPD Relative afferent papillary defect

RF Radiofrequency

RMA Robust multichip analysis

RPMI Roswell Park Memorial Institute medium for cell culture

RR Relapsing remitting

RT-PCR Real-time polymerase chain reaction

SD Standard deviation

SDS-PAGE Sodium dodecylsulphate polyacrilamide gel electrophoresis

SE Spin echo

SSEP Somatosensory evoked potentials

T Tesla

TAE Tris- acetate

TCC p38

TCL T cell lines

TCR T- cell receptor

TLR Toll- like receptor

TNF-α Tumour necrosis factor- α

T2W T2 weighted

TE Echo time

TGF-β Transforming growth factor- β

Th T-cell hlper

TMB Tetramethylbenzidine

TR Repetition time

VER Visual evoked potentials

VLA-4 very late antigen 4

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Chapter 1

Multiple Sclerosis (MS); An Overview

1.1 Introduction

MS is a chronic inflammatory immune mediated demyelinating disease of the central nervous system (CNS) in genetically susceptible individuals.

Throughout the twentieth century advances in medical research have led to an increased understanding of the aetiology and pathogenesis of MS. Epidemiological studies and molecular genetics have provided important insights. Both genetic and environmental factors may be involved in susceptibility to as well as in the outcome of MS, but their relative importance has yet to be fully quantified.

In this chapter I will present an overview of the origins, epidemiology, natural history, diagnosis and treatment of MS[1;2].

1.2 Historical overview

Over the centuries, personal accounts of what it is to have MS, were written by artists, poets, authors and ordinary people. As early as the 14 century, St Lidwina of Schiedam in the Netherlands was reported to have symptoms consistent with MS, however the first officially reported case of MS was that of Augustus D'Este a young English nobleman born on the 13th of January 1794. In December 1822, Augustus D'Este-, traveled to Scotland to visit a relation whom he found to be dead on his arrival. Shortly thereafter, D'Este experienced

loss of vision in both eyes, which recovered but which reoccurred in 1826. Further similar attacks followed and in 1843 and after a long course of relapsing remitting illness, D'Este entered a progressive disease course with superimposed relapses. Later, he became paralyzed and died in December 1848, having had a neurological illness for 26 years [3;4].

Although a detailed description of MS was written by the clinico-pathologists Robert Carswell (1838) and Jean Cruveilhier (c 1841), it was Jean-Martin Charcot who in 1865 at Salpétrière Hospital gave a definitive account of MS as a distinct disease *"La sclerose en plaque disseminees"* [5]. However, Eugéne Devic (1858-1930) described the combination of myelitis with bilateral optic neuritis, which was later termed as neuromyelitis optica (Devic's disease) [6]. The disease was first described as a single episode of spinal cord and optic nerve or chiasmal disease occurring in any order. Since then, epidemiological and pathological studies of demyelinating disease have clearly shown that neuromyelitis optica is a distinct disease and probably unrelated to MS.

Between 1920 and 1950, MS was believed to be a rare condition, however this insight has since changed dramatically. By the 1950s, neurologists considered MS to be the most common neurological disease in young adults in America. This increase in prevalence may largely be attributed to the increase in the number of trained neurologists and altered concepts of gender and disease. Now MS is diagnosed more frequently because there are more trained neurologists who have the skills necessary to make this difficult diagnosis. In the past, a significant number of patients with MS were misdiagnosed with other diseases

such as hysteria and neurosyphilis; but increased knowledge and advanced technology have facilitated their subsequent correct diagnosis [3]

In 2004, Alastair Compston published a lecture (The marvellous harmony of the nervous parts': the origins of MS) which was based on the Croonian lecture (William Croon 1664- *De ratione motus musculorum*) [4]. In this article he gives a superb account of the origins of MS and explains the evolutionary hypothesis of the origins of MS as it evolved from a disease already present in more ancient populations, namely, neuromyelitis optica.

1.3 Epidemiology of MS

The uneven worldwide distribution of MS led to the extensive investigation into the epidemiology of MS. In 1993, Kurtzke classified different regions of the world according to their prevalence of MS. High prevalence regions had more than 30 cases of MS per 100,000 population, intermediate prevalence had 5-30 and low prevalence had less than 5 cases of MS [7].

Ireland is considered to have a very high prevalence of MS. Reports from Northern Ireland published initially in 1954 by Allison and Miller on a population of 1,370,709 gave a prevalence of 51 per 100,000 [8]. Further studies by G. V. Mc Donnell and S.A. Hawkins in the Royal Victoria Hospital (Belfast, Northern Ireland) on a sample population of 151,000 showed a prevalence of 168.2/ 100,000 (95% CI, 148 to 189/10^5) [9]. The estimated number of patients with MS in the Republic of Ireland now is about 6,000. It is a disease affecting young people hence it represents a significant burden to healthcare providers.

1.3.1 Migration Studies

Geoffrey Dean carried out one of the earliest and most convincing studies of migrants in South Africa. In 1967, he gave an account of the annual incidence, prevalence and mortality statistics for MS in white South African- born individuals and immigrants to South Africa [10]. He also addressed the issue of the marked difference in frequency of MS depending on age of arrival to South Africa in the English speaking white population. Migrants under the age of 15 showed lower frequency as compared to the older immigrant population. He had arrived in Cape Town and was very much surprised by the absence of MS in African blacks, whereas the rate was higher in the mixed race population (African and Caucasians). Since then numerous studies have been carried out on migrants in different countries. In the UK in 1997, Dean and Elian studied 76 non- Caucasian immigrants from India, Pakistan and Bangladesh, who were born in the Indian subcontinent, east Africa, Fiji or Malaysia and had MS [11]. A higher incidence than expected was observed in individuals arriving in the United Kingdom under the age of 15. Conversely, this did not apply to the 60 patients with MS born in the Caribbean.

In 1995 Gale *et al* reviewed the literature on migrant studies, comparing rates of MS in migrant populations with those in the host country and in the country of origin. In their review they pointed out the difficulties associated with interpreting and drawing conclusions from such studies. Migrating individuals rarely represent their original general population. They are often younger, healthier and of higher socioeconomic status. Therefore, the quality of the data

produced by such studies may be poor and add little to our understanding of the genetics of MS but they emphasise the importance of environmental factors [12]. However, they supported studies that showed that individuals migrating from a high prevalence area to an area of low prevalence tend to adopt the low risk of the new country. This is in contrast with those who migrate in the opposite direction who tend to retain the low risk of their country of origin [13].

A correlation between the age of migration and the risk of MS has also been studied. Individuals migrating from a high risk area to a low risk area before the age of 15 acquire the risk of the new area, whereas individuals migrating after the age of 15 retain the risk of the area of their origin [14].

1.3.2 Racial factors

MS is rare among the indigenous black people of Africa. In 1987, the first account of a black patient with MS in South Africa was published. In 1994, Dean *et al* published seven cases of black patients in South Africa and five in Zimbabwe in whom a diagnosis of MS was established. Six of the 12 patients had severe optic neuritis and subsequently became blind. Clinical features in these few black patients resembled those seen in oriental patients affected by MS rather than those seen in white south Africans or the black people of North America or the Caribbean, indicating a real difference in the phenotype of the disease in the population [10]. Approximately 6% (2-16%) of North American MS patients have an affected family member where as in Asia and Chile, the figure is much lower. In 1979, Kurtzke *et al* studied 5305 World War II and Korean conflict veterans who were diagnosed with MS and matched to controls on the

basis of age and date of entry into military service. Their findings suggest that a racial and genetic predisposition, as well as a geographically determined differential exposure to an environmental agent, are related to the risk of MS [15]. Analyses of Scandinavian co affected sibling pairs carried out in 2004 by Oturai *et al* on 136 Caucasian Scandinavian families suggest that disease course and age of onset are partly under genetic control. Furthermore, HLA-DR2 in probands of sibling pairs suggests importance for age of onset [16].

1.3.3 Age, gender and phenotype

MS affects mainly young adults with the peak age at onset of around 30, and it shows predominance for female with a ratio of 2:1 irrespective of ethnicity, but this gender difference changes with the age at onset [17;18]. A very early onset of the disease has been described in children under the age of six with even higher female predominance of 3F:M [19]. The youngest of them was a ten-month-old girl. For patients under thirty years of age, the disease is typically characterised by periods of inflammation (relapses) and variable periods of remission (complete or incomplete remission): a relapsing remitting pattern [20]. Fifteen to twenty five years after the first clinical onset, seventy percent of untreated patients enter the progressive phase known as secondary progressive MS [17].

In patients over fifty years of age, the disease often manifests as primary progressive MS, characterised by continuous progression of neurological disability from the initial onset of the disease, with only occasional plateau or temporary minor improvements [21;22]. In 1983, Noseworthy *et al* studied a large

MS population (N = 838) of which 9.4% of the patients had late onset MS. The pattern of the initial symptoms and subsequent course of the disease in the older patient group was characterised by slow deterioration of motor function compared to that in the younger patient group. Also laboratory studies such as evoked potential studies and cerebrospinal fluid (CSF) electrophoresis were of high diagnostic yield in the older patient group [23].

1.3.4 Seasonal variations

It has been suggested that environmental factors play an essential role in the aetiology and pathogenesis of MS. Studies have demonstrated a relationship between seasons of the year and "outbreaks" of MS. Abella-Corral *et al.* followed up 31 patients over a period between 1997 and 2002 and calculated the monthly and quarterly rate of incidence of outbreaks [24]. A higher incidence of outbreaks in the summer months (more in June) and a lower incidence in winter (less in December) were observed. This study indicated outbreaks of MS might be related to seasonal variations, with a higher number in the warmer months and fewer in the colder months. Eight hundred and thirty four patients with late onset MS residing in Washington and California were followed up over a 20 years period. Worsening disease, was reported in those exposed to hot climate [22]. Recent studies suggest that exposure to sunlight and high levels of vitamin D in the serum reduces the risk of developing MS [25].

1.3.5 Latitude variation and prevalence of MS in Ireland

Ireland is recognised as a high-risk area for MS. A study in the Republic of Ireland by Brady in 1977 reported a prevalence rate of 77/100 000 [8]. The most

recent study conducted in the north east of the island reported a prevalence rate of clinically definite MS of 168.2/100 000, one of the highest prevalence figures within Western Europe [9]. Latitude based variation in the prevalence of MS has previously been reported in other regions of the British Isles and worldwide [26] [27].

A recent epidemiological study on MS in the Republic of Ireland examined whether the prevalence in MS differs between two counties is explicable on the basis of latitude variation and genetic difference between the two populations predisposition to MS. The study included seventy-three MS patients from County Donegal and forty-five from County Wexford with 200 control subjects enrolled from each county. County Donegal in the north west of the country had a higher prevalence rate than County Wexford in the south east of the country (184.6/100,000 vs. 120.7/ 100,000). County Donegal showed a significant prevalence difference of 63.9/100,000 (95% CI 49.3-82.7/100,000) between the two regions (Z = 3.94, p0.001) [28]. The study also confirmed the strong association between the HLA phenotypes: DRB1*1501 and DQB1*0602 and the development of MS in both patient populations relative to controls. It also showed that the Donegal sample control population had a significantly higher carriage rate of the HLA DRB1*1501 - DQB1*0602 haplotype relative to the Wexford sample control [29].

1.4 Natural History

The natural history of MS is unpredictable and has been examined and discussed for several decades. Overall, the clinical course of the disease is made up of two distinct types of neurological episodes, exacerbations and progression. An

exacerbation is the reappearance or worsening of neurological symptoms lasting more than 24 hours with episodes separated by at least one month. A relapse is usually followed by a remission although in some cases concludes with permanent neurological deficit. Fatigue alone or transient worsening of symptoms associated with increased body temperature, infection or stress are not considered as exacerbations.

Progression is defined as a continuous neurological deterioration for a minimum of six months. There is no limit to the worsening of symptoms but some neurologists consider an increase of 0.5 in the Kurtzke EDSS scale as a definite progression (Table 1.1) [30]. Once progression starts, it continues, although patients may get temporary remission. The start of progression is assessed retrospectively.

Two clinical onsets of MS are seen, one that starts with an initial attack followed by remission and one that initially starts as progressive with or without superimposed relapses. In 1991, Weinshenker *et al* suggested that several factors at presentation were significantly associated with an adverse outcome including older age at onset, male sex, cerebellar involvement or insidious onset of a motor deficit as first symptom. Other factors also associated with a poorer outcome included persisting deficits in brainstem, cerebellar or cerebral systems, a higher frequency of attacks in the first 2 years after onset of disease, a short first inter-attack interval and higher DSS at 2 years and 5 years from onset [31].

Survival is not greatly shortened in mildly disabled patients, but the observed mortality is increased four-fold over the general population in patients with advanced disability [32].

Table 1.1 Kurtzke Expanded Disability Status Scale

0.0	Normal neurological examination
1.0	No disability, minimal signs in one FS
1.5	No disability, minimal signs in more than one FS
2.0	Minimal disability in one FS
2.5	Mild disability in one FS or minimal disability in two FS
3.0	Moderate disability in one FS, or mild disability in three or four FS. Fully ambulatory
3.5	Fully ambulatory but with moderate disability in one FS and more than minimal disability in several others
4.0	Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability; able to walk without aid or rest some 500 meters
4.5	Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability; able to walk without aid or rest some 300 meters.
5.0	Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (work a full day without special provisions)
5.5	Ambulatory without aid or rest for about 100 meters; disability severe enough to preclude full daily activities
6.0	Intermittent or unilateral constant assistance (cane, crutch, brace) required to walk about 100 meters with or without resting
6.5	Constant bilateral assistance (canes, crutches, braces) required to walk about 20 meters without resting
7.0	Unable to walk beyond approximately five meters even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone; up and about in wheelchair some 12 hours a day
7.5	Unable to take more than a few steps; restricted to wheelchair; may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day; May require motorized wheelchair
8.0	Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms
8.5	Essentially restricted to bed much of day; has some effective use of arms some self care functions
9.0	Confined to bed; can still communicate and eat.
9.5	Totally helpless bed patient; unable to communicate effectively or eat/swallow
10.0	Death due to MS

FS: functional status

(The table represents the scoring system in terms of clinical progression of MS)

The following sections include detailed account of each MS clinical phenotype.

1.4.1 Clinically isolated syndrome (CIS)

CIS is the first presentation in 85% of young adults diagnosed with MS. The clinical onset is with an acute or sub acute episode of neurological disturbance caused by a single white matter lesion, affecting the optic nerve, brainstem, or spinal cord. 30-70% of patients with CIS develop MS. MS can be diagnosed within three months of CIS presentation with certain magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) criteria. In 1989, Miller *et al* prospectively followed clinical status and MRI imaging in patients who presented with clinically isolated lesions of the brainstem or spinal cord. MS progression occurred in 13 brainstem syndrome patients (57%) and in 14 spinal cord syndrome patients (42%) after mean intervals of 15 and 16 months, respectively. Oligoclonal bands (OCB) in the CSF increased progression risk in patients with both syndromes while the presence of disseminated brain lesions, increased risk only in those with a spinal cord syndrome [33;34].

1.4.2 Relapsing remitting MS (RR MS)

In RR MS patients experience an initial exacerbation followed by complete or incomplete recovery. 80-90% of patients with MS have a RR course in the ten years after initial presentation. The disease begins with an initial attack followed by a period of remission that could be as long as two years; a second attack is followed by either another period of remission or by progression and this is termed secondary progressive MS (SP MS). Eventually, remissions are of

shorter durations, relapses become longer and finally the patient enters a progressive phase [35].

1.4.3 Benign MS

Twenty to forty per cent of MS patients have "benign disease," in which less than moderate disability is present after 10 years. Hawkins et al studied 259 patients with MS, of these, 181 had MS for more than ten years and thirty-six had benign disease (EDSS<3.0) (table I) greater than 10 years after onset. They concluded that patients with benign MS were predominantly women and younger and that sensory symptoms were most common at onset followed by optic neuritis [36]

1.4.4 Secondary progressive MS (SP MS)

50% of patients presenting with RR MS will eventually develop gradual progression of disability that may or may not be accompanied by exacerbations [37].

1.4.5 Primary progressive MS (PP MS)

10-20% of patients diagnosed with MS experience a gradual progression of disability from the time of onset that is not accompanied by exacerbations [17].

1.4.6 Relapsing progressive MS

This term describes patients who experience MS that is progressive from the time of onset and is later accompanied by one or more relapses.

1.5 Clinical features

1.5.1 Cranial nerve dysfunction

1.5.1.1 Impairment of the visual pathways

Optic neuritis (ON) is the most common presentation of first attack of MS. The usual presentation is one of pain in the eye accentuated by ocular movements, are often followed to a variable degree by visual loss (scotoma) affecting mainly central vision or impairment of colour vision as detected by the Ishihara plates. Bilateral ON occurs in some cases of MS. Recurrence of monocular or binocular ON is highly variable. Recent long-term follow-up data show that most patients with demyelinating optic neuritis have an excellent prognosis for recovery of central visual acuity.

Mapping the visual fields reveals a central or cecocentral scotoma. Bitemporal hemianopia is rare and, if present, should raise the suspicion of chiasmatic compression. Patients with ON have a positive "swinging light test" indicative of a relative afferent papillary defect (RAPD) (Marcus Gunn pupil). The interpretations of the examination findings becomes more difficult as the degree of the optic nerve impairment worsens. Disc oedema (papillitis) due to acute lesions of the optic nerve head may be present. More often, the lesion of the optic nerve is retrobulber, and therefore fundoscopic examination appears normal early on. Later, the optic disc becomes pale as a result of axonal loss and resultant gliosis. After an acute attack, 90% of patients regain their vision, typically over a period of 2-6 months. Patients often report desaturation of bright colours, particularly red, and some report a mild non-specific dimming of

vision in the affected eye. Homonoymous field defects can be seen in MS caused by involvement of the optic radiations.

1.5.1.2 Impairment of ocular motor function

Individual ocular motor nerve impairment is unusual in MS. When present, the frequency in a decreasing order is cranial nerve VI, III, and, rarely, IV. Their involvement usually presents with diplopia, which usually remits. Nystagmus is common in MS. Acquired pendular nystagmus is often characteristic of MS, in which there is rapid, small amplitude pendullar oscillations of the eye in the primary position. Patients often complain of oscillopsia. Internuclear opthalmoplegia (INO) is an abnormal horizontal ocular movement with lost or delayed adduction of the affected eye and horizontal nystagmus of the abducted eye. It is due to a lesion in the medial longitudinal fasciculus on the side of the diminished adduction. Convergence is preserved. When present bilaterally, it is usually coupled with vertical nystagmus on upward gaze. Ocular pursuit movements are often saccadic rather than smooth. Ocular dysmetria may coexist with other cerebellar dysfunctions and other ocular oscillations, such as intrusive saccadic movements (square wave jerk).

1.5.1.3 Impairment of other cranial nerves (excluding III, IV and VI)

Cranial nerve dysfunction is usually due to an upper motor neuron lesion (pseudobulbar syndrome) in MS. Subjective or objective impairment of facial sensation is a quite common finding. Trigeminal neuralgia is often one of the first symptoms of presentation of MS in a young adult. Facial myokymia is a

fine modulating wavelike facial twitching and hemifacial spasm can be one of the symptoms of MS. Unilateral facial paresis can occur. Vertigo can occur in 30-50% of patients with MS, and is commonly associated with dysfunction of other adjacent cranial nerves. Symptoms are often of hyperacusis or hypoacusis, facial numbness and diplopia.

1.5.2 Impairment of sensory pathways

Sensory symptoms are present in almost all patients with MS at some stage of their disease. They frequently manifest as the first symptoms at diagnosis. Sensory features can be spinothalamic, posterior column, or dorsal root. The symptoms are of numbness, tingling, pins and needles, tightness, coldness or swelling of limbs or trunk. It can be radicular pain, either unilateral or bilateral, often of the lower thoracic and abdominal regions. It can present as an itchy sensation often in the distribution of the cervical dermatomes.

On clinical examination, the findings may vary in the degree of impairment of vibration sense, joint position, decrease of pain and light touch in a distal distribution of the upper and lower limbs, and patchy distribution in the limbs and trunk.

1.5.3 Impairment of motor pathways

Cortical tract dysfunction is common in MS. Paraparesis or paraplegia is common occurrence and can present with severe spasticity, with extensor or flexor spasms of the legs and trunk that may be provoked by active or passive attempted movement. Spasticity is usually more marked in the lower than the

upper limbs. Deep tendon reflexes are exaggerated, sometimes with clonus and positive extensor plantar response (Babinski). Sometimes the deep tendon reflexes are reduced and this is because of lesions interrupting the reflex arc at a segmental level. Amyotrophy, if present, is usually due to disuse muscle atrophy, and usually affects the small muscles of the hands.

1.5.4 Impairment of brainstem function

Impairment of ocular motility is frequently seen in MS. Nystagmus, usually horizontal, occurs quite frequently in some studies up to 40-70% of patients may be affected. Other types of nystagmus can also occur, but to a lesser extent. Nystagmus is usually asymptomatic, but some patients may complain of blurred vision, oscillopsia (jumping image), or diplopia (double vision). Dysarthria is also commonly seen especially in chronic, advanced cases [38]. Nasal speech can occur due to IX and X nerve involvement. Blepharospasm is often associated with brainstem lesions as is facial myokymia (undulating, wavelike fascicular twitching), usually beginning in the orbicularis oculi muscle [39]. Hearing loss and vertigo are also common [40].

1.5.5 Impairment of cerebellar pathways

Impairment of cerebellar pathways may result in ataxia (gait imbalance), clumsiness, difficulty in performing coordinated movements and slurred speech. Examination reveals dysmetria, decomposition of complex movements, and hypotonia, most commonly in the upper limbs. Walking is impaired. Intention tremor of the head and upper limbs may be present. Nystagmus, ocular dysmetria, and failure of fixation suppression suggest cerebellar or

cerebellovestibular connection dysfunction. Speech can be scanning or explosive in character.

1.5.6 Impairment of bladder, bowel and sexual dysfunction

Bladder dysfunction is common and disabling finding in MS [41]. It manifest in either failure to store urine, failure to empty the bladder adequately [42;43]. The extent of the impairment often parallels the degree of motor impairment in the lower extremities. Urgency is one of the most common complaints. It is caused by uninhibited detrusor muscle contraction. As the disease progresses, urinary incontinence becomes more frequent. With sacral spinal involvement, there is decreased urinary flow, interrupted micturition and incomplete emptying. An atonic dilated bladder that empties by overflow results from loss of perception of bladder fullness and is often associated with anal and genital hypoesthesia.

Constipation is more common than faecal incontinence [44 45]. Sexual dysfunction is common symptom in MS. *Erectile* dysfunction is common so is problems with ejaculation [41]. Sexual dysfunction is reported significantly more often by men but also by women with lower EDSS scores.

1.5.7 Autonomic disorders

Autonomic dysfunction other than bladder and bowel may be seen in patients with MS. Abnormal sweating has been described, and some patients have coldness and discoloration of the legs and feet [46]. Bilateral diaphragmatic paralysis resulting in respiratory failure in MS is rare [47;48].

1.5.8 Cognitive and psychiatric symptoms

Subtle abnormality of cognitive function is quite common but frank dementia is rare. Difficulties with memory, attention and concentration, and conceptual reasoning/ problem solving are frequently seen [49;50 51]. Euphoria has been described in MS, but depression is more recognised [52;53].

1.5.9 Fatigue, sleep and pain

Seventy eight per cent of patients with MS complain of fatigue, and it is one of the most disabling symptoms experienced by MS patients [54;55]. Fatigue interferes with the patient's daily activity, work, social and family life [56]. This type of fatigue must be distinguished from depression, side effect of medication, or physical tiredness. For some patients with MS, sleep disturbance and accompanying increases in depression may be a function of the lesion site resulting in nocturnal spasms [57]. Chronic pain is a common feature of well-established MS and is usually associated with a myelopathy and therapy must be individualized for each specific pain syndrome.

1.5.10 Paroxysmal symptoms

Trigeminal neuralgia is the most common paroxysmal symptom in MS. It occurs at an early age of onset of MS and frequently bilateral [58]. Hemifacial spasm may occur and it may be caused by a lesion in or near the facial nucleus [59]. Also tonic seizures may occur in MS [60;61].

1.5.11 Movement disorders

Movement disorders other than cerebellar tremor are rare in MS. Dystonia, hemiballismus, and myoclonus can occur [62-65]. Micrographia has been reported in patients with parietal white matter lesions [66]. In 1988, Mao CC et al reported two cases of parkinsonism and a case of chorea associated with MS [67;68]

1.5.12 Clinical features distinctive of MS

L'hermitte's phenomen is a transient sensory symptom described as an electrical shock radiating down the spine or into the limbs on neck flexion [69;70]. Although most frequently seen in MS, it is also seen in other neurological diseases such as cervical cord lesions.

Uthoff's phenomena a transient worsening of symptoms due to increase of body temperature [71]. The underlying mechanism is thought to be a temperature-dependent conduction block occurring in demyelinated, and thus poorly insulated fibres [72]. It often manifests as optic neuritis (ON) or it may be a non-visual symptom such as paresthesia or weakness [73].

1.6 Supporting tests in the diagnosis of MS

1.6.1 Magnetic resonance imaging (MRI) in MS diagnosis

Even though nuclear magnetic resonance (NMR) was developed as an imaging technique in the 1940s, imaging humans became possible in the 1970's with the use of large bore magnets. Shortly thereafter in 1981, the first inversion recovery scans were made of the brain of eight clinically definite MS patients. These scans demonstrated focal areas of abnormality around the ventricles.

Since then and in the past 10-15 years, MRI scanning has played a pivotal part in the diagnosis and management of MS.

MRI technique is based on the concept that protons, such as positively charged hydrogen atoms (H^+) discharge signals that can be processed into images. These H^+ atoms (protons) are quite abundant in the extracellular water, lipids, and other molecules in body tissues. Placing an individual in a magnetic field of an MRI machine results in the alignment of these protons with the longitudinal axis (z) of the magnet in the equilibrium state. Depending on the strength of the magnetic field, these protons wobble around the z-axis in a process known as precession. The precession frequency can be calculated using mathematical equation (Larmor equation).

A radiofrequency (RF) pulse is transmitted through the head coil in order to cause those protons to move from their equilibrium position, making them rotate from the longitudinal z axis towards the horizontal (XY) axis. A 90-degree RF pulse rotates the proton into the XY plane, whereas a 180- degree pulse produces twice the degree of rotation. Turning off the 90-degree RF pulse causes a re-growth of the longitudinal magnetization along the z-axis (T1 relaxation) and loss of the transverse magnetisation (T2) with time.

The T1 and T2 relaxation times reflect the environment in which the protons reside. Free water and cerebrospinal fluid (CSF) have long T1 and T2 relaxation times, whereas fat has short T1 and long T2 relaxation time, grey and white matter have intermediate.

The pulse sequences used for MR are: spin-echo (SE), gradient-recalled echo (GRE), and inversion recovery (IR). They are a combination of RF and magnetic field gradient pulses used to create an MR image. SE imaging is more favoured in MS, it produces pulse sequences that favour or are "weighted" towards one of the relaxation times hence, T1- weighted or T2- weighted. The weighting is a reflection of repetition time (TR) and echo time (TE). T1-weighted images have a short TR and TE, whereas T2-weighted images have a long TR and TE. Proton density weighted images are produced from a mixture of T1 and T2- weighted images using long TR and a short TE.

The diagnosis of MS is based on clinical assessment, but undoubtedly, MRI scanning is the most sensitive paraclinical test available, especially with the use of higher-strength magnet and fluid-attenuated inversion recovery (FLAIR) imaging. The majority of MS patients have ovoid lesions with well-defined margins asymmetrically located perpendicularly (Dawson's fingers) to the corpus callosum, in the corpus callosum, or in the subcortical white matter, middle cerebellar peduncle, pons, or medulla [74].

1.6.1.1 T2W imaging in MS

T2W sequences have long TR and TE, therefore the CSF looks bright, and MS plaques look hyperintense. T2W images is highly sensitive in depicting focal white matter lesions, particularly in the centrum semiovale and adjacent to the ventricles.

1.6.1.2 Proton-density images

They provide similar information as T2W images in MS, but adding certain advantages in term of contrast with CSF and scan time. They are often obtained with long TR and a short TE. MS lesions are hyperintense on proton-density images, but the CSF is more hypointense (darker) than on T2W images, and therefore the border between the ventricles and periventricular lesion is more evident.

1.6.1.3 Fluid-attenuated inversion recovery (FLAIR)

It is the most sensitive imaging sequence for white matter abnormalities, it detects 2-3 times the number of lesions seen on T2W imaging. On the other hand, it is more prone to artefacts, particularly from vascular structures, and is relatively insensitive to lesions in the posterior fossa.

1.6.1.4 Contrast-enhancing imaging

Adding a paramagnetic substance such as gadolinium shortens the T1 and T2 relaxation times of the tissues. In MS, the blood-brain barrier (BBB) is breached therefore gadolinium diffuses into the extracellular space of the abnormal brain parenchyma. This in turn then alters the relaxation time of the tissue resulting in enhanced or hyperintense image production on the T1W spin echo. This is the most sensitive method to detect new active lesions with BBB breakdown [75]. The enhancement of new lesions can be detected up to one month in the cerebellum, and for an even longer period of time in lesions of the spinal cord and occurs most commonly in relapsing remitting MS.

1.6.2 Cerebrospinal fluid (CSF) analysis in MS

In 1925, Greenfield and Carmichael used CSF examination for the diagnosis of syphilis. It preceded neuroimaging and evoked potentials by decades as the first method of providing laboratory confirmation of a clinical diagnosis of MS. Since then, study of the CSF has played an important part in the investigation of the aetiology and pathogenesis of MS, due to its direct contact with the CNS.

Immunoglobulins (Ig) are produced by plasma cells that arise from clones of B-lymphocytes. Kabat *et al,* in the 1950s, showed that the proteins in the CSF of MS patients were different from those in the serum by using serum electrophoresis techniques [76-78]. Lowenthal *et al,* in the 1960s demonstrated the importance of the bands that are present in the Ig region of the CSF of MS patients in the diagnosis of MS [79].

The elevated CSF IgG in comparison to the blood IgG is due to increased synthesis of IgG in the CNS, which is also disproportionately increased in comparison to the CSF albumin, this is highly characteristic of MS. In some patients IgM and IgA are also elevated. To interpret the ratio of IgG and albumin in the CSF, it is also necessary to quantitate IgG and albumin in the serum:

$$\text{CSF IgG index} = \frac{\text{CSF IgG / CSF albumin}}{\text{Seum IgG / serum albumin}}$$

A ratio of 0.7 or higher indicates IgG is being synthesised in the CNS [80].

Oligoclonal bands (OCB) are found only in the CSF, and not in the serum, in 90% of patients diagnosed with MS [81]. They are the distinct bands demonstrated by the separation of IgG by electrophoresis or isoelectric focusing. Oligoclonal IgM and IgA may also be noted in CSF of MS patients, but to a lesser extent than IgG [82;83]. Elevated immunoglobulins are also seen in other conditions such as syphilis, subacute sclerosing panencephalitis, Lyme disease, etc [84].

Normally, very few T lymphocytes are present in the brain, making it almost undetectable by immunohistochemical methods. In MS, these cells increase in number and are readily detectable in the brain parenchyma. All the CSF lymphocytes are ultimately derived from the peripheral circulation via transmigration across the BBB. However, the distribution of lymphocytes subsets in the CSF is different from that of peripheral blood. In the CSF, at least 80% of T lymphocytes express CD3 compared with only 65% T lymphocytes in peripheral blood. The ratio of CD4+ (T- helper) to CD8+ (T- suppressor) remains at 2:1 as it exists in peripheral blood. CSF- derived T lymphocytes demonstrate enhanced adhesion to vascular endothelial cells, they also express a greater number of interleukin-2 (IL-2) cell surface receptors and have increased mRNA and DNA synthesis, suggesting cellular activation.

1.6.3 Evoked potentials in the diagnosis of MS

Certain stimuli cause the nervous system to generate electrical potentials called Evoked potentials (EPs). These electric potentials have been used in the study of

the pathophysiology of demyelination and as an adjunct in MS therapeutic trials. They have been used for the past 25 years in the diagnosis of MS.

EPs are sensitive and objective and can detect silent lesions. They represent electrical potentials (voltage) that are evoked by brief sensory stimuli. The impulses are conducted along the peripheral and central nervous system pathways. Delay or blockage is detected when they cross through a demyelinated region. The EPs generated from beyond the demyelination are abnormal because they are delayed, attenuated, or absent. EPs can only test certain areas of the CNS: the central visual pathways, the brainstem auditory pathways, and the posterior column/ medial lemniscus/ internal capsule sensory pathways.

1.6.3.1 Visual evoked potentials (VEP)

It was first described by Richey et al in 1971 [85]. A pattern reversal device is used to determine visual evoked potentials. It comprises a checkerboard of black and white squares, in which each black square becomes white and each white square becomes black twice in one second. This is displayed on a television screen controlled by a small computer or with a mirror and a galvanometer. The test is carried out in a dark room, and one eye is tested at a time. Recordings are made over the occipital scalp. Hundred separate stimuli are given, and the results are averaged. The positive polarity peak seen at 100 ms is called P100, and is achieved by adding a series of neurological events. For example the potentials are conducted from the eye along the optic nerve, across the chiasm, and down the optic tract to the lateral geniculate body and from there via the

optic radiation to the occipital lobe, finally, a large positive peak is generated from the striate cortex and detected at the occipital scalp as the P100 peak. In MS, impairment of conduction may occur at any point along the pathway. VEPs are more sensitive to detect demyelination than clinical examination. Once a lesion is present with prolonged VEP, very little improvement is expected even after many years [86]. Therefore, VEPs are important in detecting visual changes that are due to an episode of optic neuritis that has occurred years ago.

1.6.3.2 Somatosensory evoked potentials

A brief electrical stimulus is applied to the median nerve at the wrist, or the posterior tibial nerve at the ankle. Recordings are then taken from electrodes located peripherally over the brachial plexus or the lumbar spinal cord. For the median nerve, the principle peaks detected are generated at the brachial plexus, midcervicomedullary junction or nearby the thalamus, and finally at the rolandic fissure. For the posterior tibial nerve, potentials are detected at the lumbar cord and the rolandic fissure. The pathways underlying SEPs are the posterior columns, medial lemniscus, and internal capsule. Comparison of the latencies and amplitudes of the various peaks can help the examiner to interpret the anatomical levels of disruption along the pathway.

1.6.3.3 Brainstem auditory evoked potentials

This is done by the transmission of 100 stimulli/μs clicks through earphones. Sensitive pathways in the pons and midbrain are associated with the ability to localise an auditory stimulus in space rather than those used for speech and tone discrimination are detected.

1.7 Diagnostic criteria

Diagnosis of MS is based on the clinicopathological observations of Charcot and requires demonstration of lesions which are disseminated over time and which involving multiple, discrete anatomical regions of central white matter [87]. Schmacher *et al.* categorised patients as "clinically definite, probable, or possible" MS, according to the following criteria: [88]

1. Age of onset between 10 and 50 years.
2. Objective neurological signs present on examination.
3. Neurological symptoms and signs indicative of CNS white matter disease.
4. Disseminated in time:
    a. Two or more attacks, lasting more than 24 hours, and separated by at least one month or,
    b. Progression of symptoms and signs for at least six months
5. Dissemination in space: two or more non-contiguous anatomical areas involved
6. No alternative clinical explanation

Patients who met five or six of the above criteria were classified as patients with "clinically definite MS". Patients who met fewer criteria were classified as "probable" or "possible MS". Scumacher's criteria for the diagnosis of MS, was solely based on clinical history and examination.

In the early 1970s advances in neuroimaging occurred. Beginning with computed tomography (CT) followed by magnetic resonance imaging (MRI), these new imaging modalities have made possible to demonstrate anatomical lesions that are not clinically evident [89]. New MRI techniques, such as MR spectroscopy (MRS) and magnetisation transfer, are future potentials for the assessment of MS.

Elevation of immunoglobulin CSF IgG levels, increased IgG index, increased IgG synthesis rate and the presence of oligoclonal bands have aided in the recognition of MS as being autoimmune in origin [90-93].

In the 1970s and 1980s advances in technology allowed the development of evoked response testing, a measure of the electrophysiological dysfunction in the visual, brain stem auditory, or somatosensory pathways.

From the aforesaid, several researchers have proposed different diagnostic criteria. In 1988, Paty and colleagues suggested the presence of four or more lesions[94], or three lesions, of which one is periventricular, as diagnostic of MS. Fazekas and colleagues criteria requires the presence of three or more lesions with at least two of the following characteristics: an infratentorial lesion, a periventricular lesion, or a lesion larger than 6mm[95]. In 1997, Barkhof and colleagues developed criteria for the prediction of clinically isolated syndrome (CIS) developing into clinically definite MS[96]. Their selection of criteria was based on MRI assessment: the presence of at least one juxtacortical lesion, at least one gadolinium- enhancing lesion, at least one infratentorial lesion, and

three or more periventricular lesions. The presence of at least three of the four features was shown to be a more accurate predictor than the Paty or Fazekas criteria for the development of MS. Tintoré and colleagues modified Barkhof's criteria by allowing nine T2 lesions in place of the gadolinium enhancing lesion[97;98].

1.7.1 Poser's criteria

Until recently, the most widely recognised diagnostic classification of MS was known as Poser's criteria [99]. The criteria incorporated historical and clinical symptomatology and paraclinical evidence, including CSF findings (presence of OCB or raised IgG /albumin index), neurophysiological tests and neuroimaging. The classification comprised two major groups, definite and probable, each with two subgroups, clinical and laboratory supported MS.

Clinically probable MS:

i. Two attacks and clinical evidence of one lesion,

ii. One attack and clinical evidence of two separate lesions,

iii. One attack, clinical evidence of one lesion and paraclinical evidence of another, separate lesion

Clinically definite MS:

i. Two clinical attacks or relapses or relapses and clinical evidence of two separate lesions; or

ii. two attacks with clinical evidence of one lesion and paraclinical evidence of another separate lesion. Two attacks must involve

different parts of the CNS, must be separated by a period of at least a month and each must last a minimum of twenty-four hours.

1.7.2 Mc Donald criteria

The present clinical guidelines with diagnostic criteria, were published in 2001 by WI McDonald [100] (Table 1.2). This was revised in 2005 by Polman et al [101]. The guidelines, like Poser's criteria, rely on objective evidence of dissemination in time and space. The McDonald guidelines rely on the modified Barkhof-Tintoré criteria for evidence of dissemination in space.

To fulfil the criteria, the history and examination should reveal two or more episodes of neurological symptoms disseminated in time and space. Also the presence of supporting tests such as the presence of multifocal white matter lesions of different age and size seen on MRI and positive CSF analysis of oligoclonal bands (OCB) [81]. Aforesaid, patients are either diagnosed with definite MS, possible MS (for those who are at risk of MS, but with equivocal investigations) or not MS (other neurological conditions).

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks; objective clinical evidence of 2 or more lesions	None[a]
Two or more attacks; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *or* Await further clinical attack implicating a different site
One attack; objective clinical evidence of 2 or more lesions	Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
One attack; objective clinical evidence of 1 lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *and* Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
Insidious neurological progression suggestive of MS	Positive CSF[c] *and* Dissemination in space, demonstrated by 1) Nine or more T2 lesions in brain *or* 2) 2 or more lesions in spinal cord, *or* 3) 4–8 brain plus 1 spinal cord lesion *or* abnormal VEP[e] associated with 4–8 brain lesions, or with fewer than 4 brain lesions plus 1 spinal cord lesion demonstrated by MRI *and* Dissemination in time, demonstrated by MRI[d] *or* Continued progression for 1 year

If criteria indicated are fulfilled, the diagnosis is multiple sclerosis (MS); if the criteria are not completely met, the diagnosis is "possible MS"; if the criteria are fully explored and not met, the diagnosis is "not MS."

[a]No additional tests are required; however, if tests [magnetic resonance imaging (MRI), cerebral spinal fluid (CSF)] are undertaken and are *negative*, extreme caution should be taken before making a diagnosis of MS. Alternative diagnoses must be considered. There must be no better explanation for the clinical picture.
[b]MRI demonstration of space dissemination must fulfill the criteria derived from Barkhof et al[6] and Tintoré et al[7] (see Table 1).
[c]Positive CSF determined by oligoclonal bands detected by established methods (preferably isoelectric focusing) different from any such bands in serum or by a raised IgG index.[14,15]
[d]MRI demonstration of time dissemination must fulfill the criteria listed in Table 2.
[e]Abnormal visual evoked potential of the type seen in MS (delay with a well-preserved wave form).[16]

Table 1.2 Represents Mc Donald criteria for the diagnosis of MS. [100]

1.8 Treatment

MS has represented a major therapeutic challenge for neurologists since it was first described by Charcot. This challenge is in no small part due to the complex, yet unresolved aetiology. An infectious, particularly viral trigger has been proposed as a trigger for disease initiation. It has also been suggested that it is an immune- mediated disease characterised by several types of inflammatory cell types in the MS lesion with altered levels of inflammatory cytokines in the serum, CSF, and CNS. Most patients diagnosed with MS will achieve a variable degree of remission after a relapse with or without treatment. However, many will be left with clinically detectable residual neurological deficit. Also with recurrent relapses, patients may end up with substantial functional disability [102;103]

1.8.1 Pharmacological therapy

The presumption of the pathogenesis of MS being immune-mediated has led to therapeutic attempts to modify the immune system both generally and selectively. Anti-inflammatory, immunosuppressant, and immunomodulatory therapies have all been employed in the treatment of MS. More specifically, these treatments have involved the use of glucocorticoids, immunosuppressant drugs, physical agents such as irradiation and modifications of the immune environment with therapeutic plasma exchange and intravenous immunoglobulin. More recently, alteration of events surrounding antigen presentation and individual stages of the immune response including cellular proliferation, recruitment, and infiltration of the central nervous system have all been targeted for therapeutic trials. More selective approaches have with

attempted to interfere with elements of the trimolecular complex through blocking MHC class II, modifying T-cell receptor functions, interfering with co-stimulatory recognition steps and altering cytokine effects or lymphocyte adhesion. The current therapeutic trials of antigen-driven peripheral tolerance, MHC class II blockade, and immunomodulation, especially with interferon-beta, illustrate the progression from broad immunosuppressive treatment to targeting specific activities of the immune system. The combination of new strategies in immunotherapy and sensitive disease monitoring of their effects should allow for more rapid identification of beneficial and tolerated treatment for MS.

1.8.1.1 Corticosteroid therapy in MS

Studies have shown that treating MS patients with corticosteroids during acute relapse will facilitate more rapid recovery with less neurological sequelae. In addition, corticosteroids induce rapid improvement of abnormal CSF IgG synthesis rate and myelin basic protein, and also improvement of the number of plaques seen on MRI. However, it is not known if treatment with corticosteroids reduces the incremental functional disability that may accumulate after recurrent relapses of MS. Corticosteroids are potent anti-inflammatory and immunosuppressant drugs. In 1961, a randomised, placebo- controlled trial was carried out on MS patients treated with a three week tapering dose of intramuscular ACTH within fourteen days of an acute exacerbation. The trial concluded that treated patients showed significant improvement symptoms in comparison to well matched placebo- treated control patients. Ten years later, a similar but larger multicenter trial was carried out and again showed significant improvement in relapse symptoms compared to placebo. Since then, two week

treatment with ACTH became the treatment of choice for acute exacerbations of MS.

Studies had previously reported the benefits of high dose intravenous methylprednisolone therapy in patients with acute renal transplant rejections. Consequently, very high dose intravenous methylprednisolone therapy was attempted in patients with acute relapses of MS. Since then, several controlled and uncontrolled trials were carried out, and the results have clearly shown that MS patients in acute relapse treated with short-term high dose intravenous methylprednisolone improve more rapidly than placebo treated controls.

Until more effective treatments are available, it is the current practice to treat patients with acute exacerbations of MS with short-term high dose intravenous methylprednisolone provided they can tolerate the side effects. Close clinical monitoring is required and the duration of treatment depends on the individual patient's response.

1.8.1.2 Interferon therapy in MS

Interferons are a family of cytokines that posses antiviral, antiproliferative, and immunomodulating properties. They are natural human proteins: type I, (include IFN-α and IFN-β) and type II (includes only IFN-γ). The year 1993 was a marked turning point in terms of the management of MS. IFN-β was the first interferon to be approved by the US Food and Drug Administration, and 68,000 patients with MS were prescribed the drug in the United States. It was licensed

for patients with RR MS. The treatment showed a reduction in the exacerbation rate and a decrease in MS activity in the brain based on MRI findings.

The mechanism of action of IFN-β remains unclear, however studies have shown that it has anti-inflammatory properties both systemically and in the CNS. It inhibits lymphocyte proliferation, decreases antigen presentation, modulates the production of proinflammatory cytokines causing a shift of the cytokine profile towards Th2 anti-inflammatory phenotype, decreases production of tumour necrosis factor (TNF-α), and reduces T- cell migration into the CNS. The first pilot study of IFN- β-1b in MS was conducted in 1986. Since then, multiple studies have shown IFN- β-1b to be effective in reducing the rate and severity of exacerbations in patients with RR MS, as well as disability and disease progression. β- interferons that are currently used are; two IFN-β 1a (Avonex^{R} and Rebif^{R}) and one IFN-β 1b (Betaseron^{R}) [104 105;106]. All interferons induce the production of antibodies. Neutralizing antibodies have been detected in patients treated with IFN-β-1b however, deleterious negative effect of neutralising antibodies on treatment with IFN-β-1b is still doubtful.

1.8.1.3 Glatiramer acetate therapy in MS

In 1987 glatiramer acetate was approved by the US Food and Drug Administration for MS treatment and has since come into widespread use for treatment of relapsing remitting MS [107;108]. It consists of synthetic polypeptides. The principal mechanism of action of glatiramer acetate is a) interference with T-cell activation by competition with MBP for the MCH class II binding site involved in antigen presentation and b) induction of glatiramer acetate specific

T lymphocyte that produce regulatory cytokines and downregulate the disease process via the process of bystander suppression [109].

1.8.1.4 Natalizumab (Tysabri®)

In 2004, Tysabri® was approved as a new treatment for MS. It is a monoclonal antibody, which exerts its action by blocking the trafficking of peripheral T lymphocytes into the CNS by VLA-4 molecules on lymphocytes. Tysabri® has been shown to reduce the rate of relapses and progression as well as reduce the number of new lesions on MRI [110;111].

1.8.1.5 Mitoxantrone therapy in MS

Mitoxantrone is a cytotoxic anthracenedione. It has an immunosuppressive action on B- and T- lymphocyte activity. In 1998, the results of the European multicentre placebo- controlled trial of mitoxantrone in progressive MS demonstrated a beneficial effect. The common side effects are leukopenia, mild alopecia, nausea, menstrual disorders and infertility.

1.8.2 Plasmaphoresis therapy in MS

Plasmapharesis involves the removal of plasma and its replacement, generally with saline and albumin. It is used in the treatment of other neurological disorders such as myasthenia gravis where the offending immunoglobulin is known. The mechanism of its therapeutic effect in MS is not known, but it could be as a result of the removal of demyelinating immunoglobulin, proinflammatory cytokines etc.

1.8.3 Immunoglobulin therapy in MS

Intravenous immunoglobulin therapy has been used in a wide variety of disorders and stems from the success of using specific antibodies prophylactically to prevent haemolytic disease of the newborn, a process that is immunologically based. Subsequently, non-specific immunoglobulin therapy was used in patients with that are immunologically based or could have viral origin. In 1950 Alexander and colleagues carried out the first trials on MS patients. The trials were based on the theory that MS is a disease of viral aetiology and that blood or blood products of healthy subjects would contain antibodies to that agent. Since then, and with the improvement of IgG production technology, more studies were conducted. To date they have shown beneficial effect on relapse rate and disability. The side effects seem to be of minor importance provided that it is administered as a slow infusion and patients are closely monitored.

Chapter 2

Multiple Sclerosis (MS); Pathology, Disease Mechanisms and the Role of Genetic Factors

2.1 Introduction

Traditionally, MS is considered to be a chronic inflammatory autoimmune demyelinating disease of the central nervous system (CNS) in which genetic and environmental factors influence the susceptibility to the disease [17]. In 1868, Charcot described it as an inflammatory disease in which inflammatory cells accumulate in a perivascular distribution within the brain and spinal cord white matter manifesting in intermittent neurological dysfunction [4;5;112]. Since then, the disease was described as sclérose en plaques disseminée or multiple sclerosis. In 1948, Kabat provided further evidence to the inflammatory understanding of the disease by his observation of the increase in oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) of MS patients [113]. Then in 1933, Thomas Rivers at the Rockefeller Institute demonstrated the immunological nature of the disease. He repeatedly injected rabbit brain and spinal cord into primates this led to CNS demyelination and neurological deficit in the immunised animals, hence the term experimental autoimmune encephalomyelitis (EAE) [114].

Over the past few decades it became more evident that MS is largely a T lymphocyte mediated autoimmune disease [115-119]. It has also become more evident that it is a multifactorial, both genetic and environmental factors contribute to the disease process [120]. It has also been speculated that it is a

polygenic disease [121]. Hitherto, the most robust genetic association with MS has been established with alleles of the major histocompatibility complex (MHC; HLA in humans on chromosome 6p21) [122;123].

Despite significant research to date, the exact molecular mechanisms underpinning the initiation and progression of multiple sclerosis remains to be elucidated. This knowledge gap has contributed to the fact that the therapeutic options available to patients with the disease remain limited. In this chapter, I will present an overview of the current understanding of the genetics, pathological basis and the molecular mechanisms of the disease process[1;2].

2.2 Pathology of MS

The study of MS pathology has shifted from a descriptive to a more dynamic approach. This is based on the correlation between histological features observed post mortem and the preceding clinical presentation, progression, and response to therapy.

2.2.1 Demyelination

The pathological hallmark of MS is demyelination. It is caused by inflammation and is associated with the formation of focal lesions in the CNS resulting in the formation of *plaques*. It is believed that peripherally activated inflammatory cells enter the CNS due to a breach in the integrity of the blood brain barrier (BBB) in genetically susceptible individuals causing inflammation and subsequent demyelination (figure 2.1). As a result of the demyelinating process, the myelin is stripped off the axons resulting in the reduction of the conduction

velocity of the axonal action potential precipitating neurological impairment [124]. A low grade axonal damage also occur in MS plaque, periplaque, and normal appearing white matter which begins early, and likely contributes to disease progression.

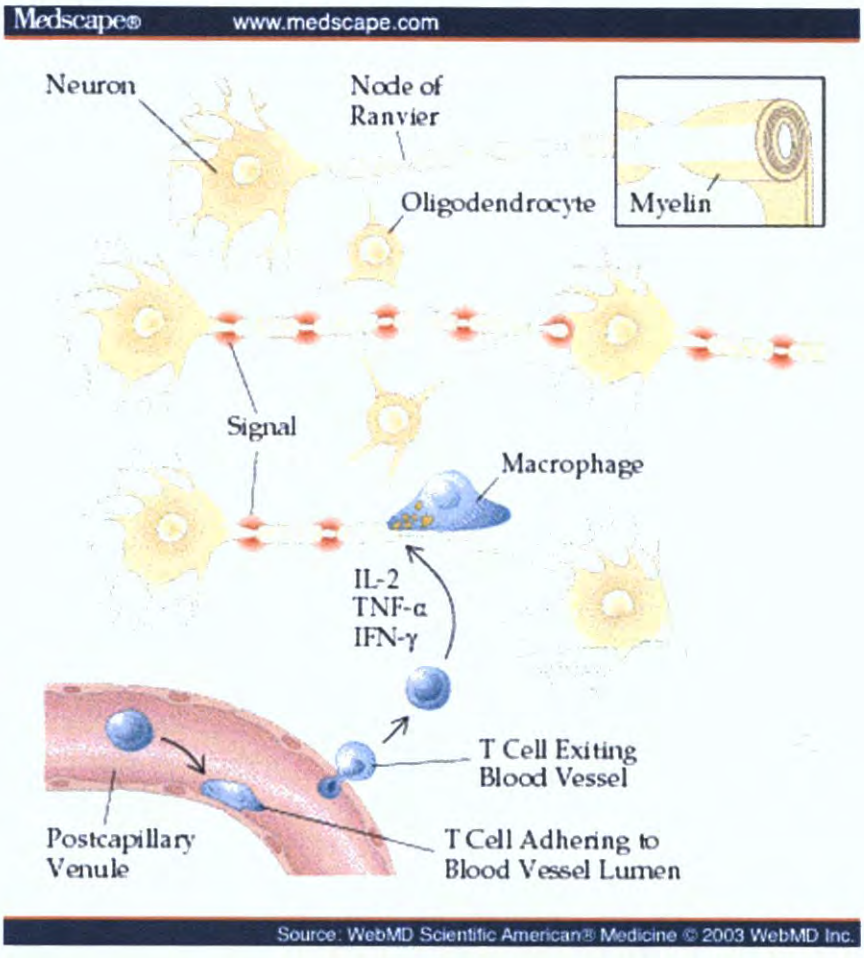


Figure 2.1. Structure of neuron and mechanism of demyelination

www.medscape.com/.../45/35/453515/art-fig1.gif

These plaques are characterised by areas of demyelination with glial scars and axonal loss. MS pathology is not restricted to the focal white matter lesions, but also includes diffuse cortical and white matter damage.

2.3 The Plaque

2.3.1 Acute plaque

The classic acute MS plaque is notable for its perivascular infiltration with mononuclear cell (T-lymphocytes and macrophages) [125] [126], with macrophages predominating. Both $CD4^+$ and $CD8^+$ T-lymphocytes as well as plasma cells are present in the plaques [127-129] [130]. Within the plaque, there is a loss of myelin and oligodendrocytes as well as axons [131] [132;133]. Often this is followed by the abundance of Oligodendrocytes precursors and progressive remyelination of new lesions can occur [134;135].

2.3.2 Chronically active plaque

In chronically active plaques, the myelin itself is seen in the form of droplets. These myelin droplets, once in contact with macrophages they are phagocytosed [136] [126]. Those plaques have sharp edges surrounded by perivascular mononuclear cuffs and myelin- laden macrophages. The centre of those plaques are hypocellular and contain naked axons embedded in a matrix of scarring (fibrous) astrocytes, lipid-laden macrophages, immunoglobulin deposition, a few infiltrating leukocytes, and virtually no oligodendrocytes [137]. Occasionally oligodendrocytes become abundant and remyelination is seen.

2.3.3 Chronic plaque

Lesions display sharp edges, astroglial scar tissue, reduced number of demyelinated axons, macrophages, and vessels with thickened (hyalinized)

walls around which occasional leucocytes are seen. These lesions contain few or no oligodendrocytes [137]

2.4 Plaque heterogeneity

Several investigators have described the pathology of the plaque as being heterogenous [138] [139]. This heterogeneity is described in four patterns seen in active lesions. The pattern recognition is based on myelin protein loss, the location and extension of plaque, the pattern of oligodendrocyte destruction and complement involvement. They vary inter-individually, but not intra-individually. All four patterns are characterized by T- lymphocyte and macrophages-dominated inflammation. Pattern I is T-lymphocyte mediated, pattern II is a T-lymphocyte plus antibody-mediated autoimmune encephalomyelitis. The other two patterns III and IV demonstrate primary oligodendrocyte destruction presumably caused by viral infection or toxin induced demyelination rather than autoimmunity. In 2000 Lucchinetti *et al* described the four following patterns:

Pattern I

- T-lymphocytes and macrophages dominate this pattern of inflammation. Other molecules such as TNF- α, IFN- γ, and free radical species also contribute to the inflammatory process.

Pattern II

- Similar to I, but antibody (mainly IgG) and compliment C9neo antigen deposition also dominate the inflammatory process, both MOG and MBP- specific antibodies are involved.

Pattern III

- Is defined by the loss of oligodendrocytes at active plaque borders. It also features loss of myelin-associated glycoprotein (MAG), whereas

other myelin proteins (such as PLP and MBP) are still present. Remyelinated shadow plaques are absent.

Pattern IV

- Resembles primary oligodendrocyte injury and apoptosis through DNA fragmentation with secondary demyelination associated with macrophages. It is least common and occurs in the PP MS. The highest extent of inflammation occurs in RR MS and in time declines with the evolution of SP MS.

Demyelinated plaques pattern I and II have sharply demarcated edges with perivenous extensions and are usually centred on small veins and venules. Pattern III; unlike I and II plaques are not centred by veins and venules, instead a rim of myelin is seen around inflamed vessels within the demyelinated plaque. The borders of the active lesions are ill defined [140;141].

Later, other researchers challenged this view. In 2004, Barnett *et al* argued the point suggesting that pattern of lesions may not be static instead an individual may have lesions featuring one or more of the above patterns[139].

2.5 Blood brain barrier (BBB)

Demyelination and plaque formation described thus far are dependent upon a loss of selective integrity of the BBB.

Significant clinical and MRI (reduction in Gadolinium enhancing lesions) trials of MS have demonstrated the role of $\alpha_4\beta_1$ integrin in the passage of mononuclear cells into the CNS. Passing through the BBB involves multiple

steps. The steps include the attachment of the mononuclear cells by binding to selectin, activation by chemoattractants, arrest and adhesion by integrin binding, and finally transendothelial activation [142].

2.6 Mechanism of axonal injury

It was Charcot, in the 19th century first described axonal injury in MS [5]. In recent years and with the development of markers it became possible to evaluate axonal injury effectively. In active inflammation such as in acute or chronic active lesions the axonal injury is seen as swellings, distortions, and truncations (spheroids) [132;133;143].

Axonal damage in MS is to some extent due to demyelination but also due to the abnormal expression of sodium channels localized within the membrane [144;145]. In order to restore normal conduction, there is an increased entry of sodium, slowing of nerve conduction and then conduction block. This is then followed by reversal of the sodium-calcium exchanger (ie. Sodium efflux and calcium influx), which can initiate intracellular cascades of calcium mediated injury, eventually leading to neuronal damage. This hypothesis has been supported by recent evidence that sodium channel blockers such as flicainide and phenytoin preserve axons in EAE [146;147 148].

In 1868 Charcot described axonal injury as being part of the MS pathology, however in 1998, Trapp documented it as an early feature of disease process. The pathological changes seen in axons are due to inflammation and the

accumulation of amyloid-precursor protein. This process continues throughout the course of the disease [149].

Factors associated with axonal damage include cytokines, nitric oxide, proteases, superoxides, CD8+T-lymphocytes, and glutamate excitotoxicity [150].

2.7 Immunology of MS

2.7.1 Cellular and molecular initiation of MS

The understanding thus far of the evolution of MS lesion involves the activation of numerous cellular and molecular pathways, ultimately resulting in demyelination and lesion formation. As previously stated the exact mechanisms remains to be elucidated, however in the following section a detailed review will be undertaken of the current knowledge pertaining the initiation and progression of the MS.

2.7.2 Overview of cellular and molecular pathways in MS

It is believed that autoreactive, myelin specific T-lymphocytes, in genetically susceptible individuals, are activated in the periphery by recognising, for example, a viral peptide or self antigen. The proinflammatory environment, in response to the viral peptide, contributes to the activation of these T cells. Factors that contribute to the proinflammatory environment are cytokines, such as IL-12 and IFN-γ, from T-lymphocytes and antigen presenting cells. The activated T-lymphocytes then adhere to the BBB endothelium via adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) and their ligands lymphocyte function

associated antigen 1 (LFA-1) and very late activation antigen 4 (VLA-4), and transmigrate into the brain parenchyma through cerebrovascular endothelial cells [151;152]

What guides these autoreactive $CD4^+$ T-lymphocytes to the CNS is still not very well understood, but the following two points are suggested: whether antigen presentation is required in deep cervical lymph nodes, which is the drainage site for brain-derived antigens and whether a chemokine gradient from inside the brain parenchyma to the blood exist during the initial event, neither suggestions are well described. Subsequently, proinflammatory cytokines such as IFN- γ, IL-23, TNF- α, and LT and chemokines such as RANTES, IP-10, IL-8 and others,

- Activate cells such as microglia and astrocytes that are resident in the CNS
- Recruit other immune cells such as monocytes, other T- and B-lymphocytes and mast cells from the peripheral blood; and
- Orchestrate the formation of the inflammatory lesion.

The formation of the inflammatory lesion is also accompanied by a breach of BBB with tissue oedema after mediators/ proteases release from mast cells, monocytes, and T-lymphocytes as well as proinflammatory molecules and oxygen and nitrogen free radicals. Damage of the myelin sheath, oligodendrocytes, and axons occurs early in the disease. $CD4^+$ autoreactive T-lymphocytes are likely driving the process. Numerous processes contribute to the myelin/ oligodendrocyte and axonal damage including free radicals, TNF- α, LT, direct complement activation and antibody dependent cellular cytotoxicity

via Fc- receptors, myelin phagocytosis, direct lysis of axons by $CD8^+$ cytotoxic T-lymphocytes, the secretion of proteases, and apoptosis of oligodendrocytes. In addition to that, the excitatory neurotransmitter glutamate that is produced by astrocytes contribute to further damage to the oligodendrocytes via glutamate receptor-mediated calcium influx. The inflammatory process lasts few days to weeks. The result is demyelinated axons, apoptotic oligodendrocytes and T-lymphocytes, axonal transactions [133] with onion bulb- like protrusions owing to interrupted axonal transport, macrophages loaded with phagocytosed myelin lipids, and the activation and beginning of proliferation of astrocytes. The next process is clearing the debris, lesion resolution and also the dominance of Th2/Th3 cytokines such as IL-10 and TGF-β, and the secretion of various growth factors such as (brain- derived neurtrophic factor, platelet- derived growth factor, ciliary neurotrophy factor, and fibroblast growth factors) by both resident cells and T-lymphocytes. Surviving oligodendrocytes present in the CNS start to remyelinate denuded internode areas. The original thickness of the myelin is usually not established. Nogo, MAG, and Omgp are inhibitory signals between axonal and myelin structures, interact with Nogo receptors that are physiologically relevant during reshaping and maintenance of the cytostructure in the CNS, delay the repair process. [153]. (Figure 2.2)

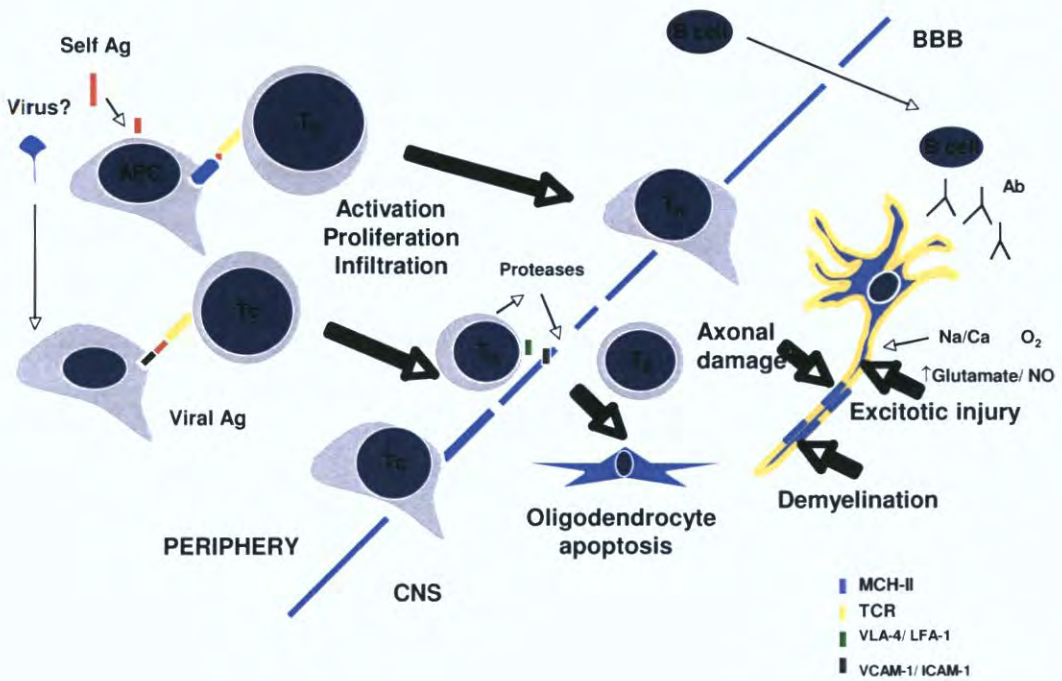


Figure 2.2. The role of the T-lymphocyte in the immunopathology of MS. Autoreactive T-lymphocyte crossing BBB from the peripheral circulation accompanied by antibodies, interacting with the microglia in CNS and its affect on the myelin.

2.8 T- lymphocytes

In 1933, Rivers showed that the injection of spinal cord or brain material into healthy primates resulted in a disease similar to MS leading to the hypothesis that MS is an autoimmune disease. Several decades later, researchers showed that the injection of an autoreactive myelin specific $CD4^+$ T-lymphocytes into rodents (experimental allergic autoimmune encephalomyelitis EAE) caused either an acute, chronic, or relapsing remitting encephalomyelitis [154 155 156]. As EAE is unlikely to be transferred by antibodies, this lead to the conclusion that it is a T cell mediated autoimmune disease. This assumption is also based on alterations seen in the blood, CSF as well as the pathologic features in the brain [157]. The following points support the above concept:

a) $CD4^+$ T-lymphocytes are part of the inflammatory infiltrate in active lesions in the CNS and CSF in MS [128 158].

b) Genetic risk is to a degree determined by HLA-DR and -DQ antigen that are recognized by $CD4^+$ T-lymphocytes [159;160].

c) The occurrence of EAE in humanized transgenic mice expressing HLA-DR or –DQ molecules [161 162;163], and the spontaneous development of EAE in mice expressing HLA-DR antigen and MS patients- derived MBP- specific TCRs [164 165].

d) Exacerbation of the disease seen in therapeutic trials using altered peptide ligand of MBP that caused cross reactivity of $CD4^+$ T-lymphocytes with Th1 phenotype [166].

e) $CD4^+$ helper T-lymphocytes, to a certain extent, control antibody production, $CD8^+$ maturation, and many other steps of adaptive and innate immune function.

2.8.1 Frequency of $CD4^+$ autoreactive T-lymphocytes

Methods of identification of autoreactive T-lymphocytes in patients with MS and healthy individuals vary greatly in terms of sensitivity depending on the technique used [167 168]. For instance, tissue culture studies show a frequency of 1 MBP- specific cell per 10^6- 10^7 PBMC [169 167] while enzyme linked immunospot (ELISPOT) assay of IFN-γ secretion in response to autoantigen is 1-2 orders of magnitude more sensitive [170]. More recent methods using quantitative real-time polymerase chain reaction (RT-PCR) have showed a frequency of $1/10^4$. Also myelin- specific T-lymphocytes with mutations of the hypoxanthine phosphoribosyl transferase gene, which occur in the proliferating T cell pool, are increased in number in MS patients during exacerbation of MS and in those that are in a treatment trial with altered peptide ligand (APL), 2000- fold expansions of MBP-specific T-lymphocytes have been described.

Studies have also demonstrated that myelin reactive T-lymphocytes from MS patients manifest a memory or activated phenotype, whereas in healthy individuals they manifest naïve phenotype [171;172]. In addition, there are lower requirements for costimulation via CD28 for MBP-specific T-lymphocyte proliferation, which is characteristic of activated or memory T-lymphocytes rather than of naïve T-lymphocytes [171;172 173]. Evidence suggesting prior activation of these myelin- reactive T-lymphocytes is evident by the increased

frequency of the hypoxanthine-guanine phosphribosyltransferase reporter gene mutation suggesting prior activation to MBP and PLP [174;175]. Also cytokine secretion and chemokine receptor class on myelin reactive T-lymphocytes of MS patients are more clearly skewed towards a cellular pro-inflammatory response [176 177].

Sospedra and Martin in their review article in 2005 demonstrated that the frequency of $CD4^+$ T-lymphocytes responding to a variety of myelin antigens is increased. There is increased secretion of IFN-γ [170] and expression of IL-12Rβ chain in absence of costimulation of T-lymphocytes [173]. During attacks there is an increase of IFN-γ and TNF-αβ secretion by T-lymphocyte clones to PLP, and increased secretion of IL-10 during remission [178].

2.8.2 Antigen specificity of myelin- specific $CD4^+$ T-lymphocytes

Myelin basic protein (MBP)

MBP is the most extensively studied myelin protein in MS. It is the second most abundant myelin protein, about 30-40% after proteolipid protein (PLP). It is relatively easy to isolate and it is used extensively to study EAE. Five isoforms of molecular weights between 14.0- 21.5 kDa are recognised, the most abundant isoform is the 18.5 kDa isoform with 170 amino acid length. It is found in the intracellular surface of the myelin membrane. It is found in significant quantity in both the central and peripheral myelin as well as peripheral lymphoid tissue. In 1999, Madsen *et al* demonstrated the encephalogenic potential of MS patient-derived T-lymphocyte in a transgenic mouse expressing a MBP- specific TCR

and HLA-DR 15[164]. EAE was induced, and about 4% of these animals developed spontaneous disease.

Proteolipid protein (PLP)

Is the most abundant CNS myelin protein, accounting for about 50% of total protein. It is highly hydrophobic and evolutionarily conserved. It is more encephalitogen compared to MBP, at least in some EAE models, particularly in SJL/J mice, in which PLP is dominant.

Myelin oligodendrocyte glycoprotein (MOG)

Is a 218 amino acid transmembrane glycoprotein of the immunoglobulin superfamily. It is much less abundant than the major myelin proteins (0.01%-0.05%). It is located in the outer surface of the oligodendrocyte membrane, this makes it accessible to antibodies as well as a target for both cellular and humoral immune responses in MS. It is expressed late in myelination and is only found in the brain/ spinal cord and the retina, not in peripheral nerves. Several studies have shown that MOG induces EAE, best examined by C57/BL6 mice in which MOG induces chronic, non- relapsing EAE.

Other myelin and non-myelin antigen as targets for $CD4^+$ T-lymphocytes

Investigators have examined the role of other myelin components and nonmyelin proteins and glycolipids as antigens for CD^+ T-lymphocytes. These include myelin- associated protein (MAG), myelin- associated oligodendrocytic basic protein (MOBP), oligodendrocyte- specific glycoprotein (OSP), α- B crystalline (α- B-C), etc.

2.8.3 CD 8 lymphocytes

Less is known about the role of $CD8^+$ T-lymphocytes than $CD4^+$ T-lymphocytes both in MS and other autoimmune diseases. This is mainly due to technical difficulties in culturing and characterisating $CD8^+$ T-lymphocyte clone. In 2003, Killestein J *et al* outlined the importance of $CD8^+$ T-lymphocytes in MS by demonstrating the increased production of lymphotoxin (LT) in SP-MS patients, increased adhesion to brain venules, an increased frequency of $CD8^+$ T-lymphocytes against EBV epitopes in MS patients, and a correlation between cytokine production by $CD8^+$ T-lymphocytes and tissue destruction reported by MRI [179].

2.9 B-lymphocytes and antibodies in MS

In 1950, Kabat *et al* illustrated high levels of immunoglobulins in the CSF of MS patients suggesting the importance of B cells and antibodies in the pathology of MS [76]. Also the evidence of humoral response is seen with increased CSF immunoglobulin during exacerbation of MS and the absence of OCB in benign MS. B-lymphocytes do not cross the intact BBB; however, once inflammation begins, B-lymphocytes, antibodies, and complement can enter the CNS. However, the immunoglobulin elevation is only seen in the CSF of MS patients and not the serum, indicates a local production in response to inflammation. B-lymphocyte activation can occur by stimulation with antigen from either self or foreign proteins, either by random bystander effect during inflammation in MS lesions, or by superantigen stimulation. Analysis of the CSF and lesions of MS patients shows increased frequency of clonally expanded memory B-lymphocytes that express variable heavy chain-4 type. The

oligoclonal bands pattern of immunoglobulins seen in the CSF of MS patients, indicates only a limited number of B cells contributes to elevations in immunoglobulin production.

B cells and antibodies play a role in pathogenesis of MS in various ways:

a) They can serve as antigen presenting cells for autoreactive T-lymphocytes.
b) They provide costimulation to autoreactive T-lymphocytes.
c) B-lymphocytes and tissue- bound immunoglobulin can recruit autoreactive T-lymphocytes to the CNS.
d) Idiotope- specific T-lymphocytes may be activated by CSF immunoglobulins, and these T-lymphocytes sustain B-lymphocytes that produce such idiotopes.
e) The most important role of B-lymphocytes is the production of myelin-specific antibodies and the destruction of myelin within plaques.

Antibodies can cause demyelination by opsonisation of myelin for phagocytosis and also via complement- mediated cytolysis.

2.10 Innate immune mechanisms in MS

This involves the recognition of conserved molecular structures produced by microbial pathogens via toll-like receptors (TLRs), cells such as macrophages, neutrophils, and mast cells, and mechanisms such as the production of lysozyme, lactoferrin, phagocyte oxidase, and nitric oxide. Also the recognition of molecular structures expressed only on normal cells, for which their purpose is to have those cells recognised as normal, and inhibit immune activation

[natural killer cells (NK cells), complements, and receptors of the C- type lectin family]. Although the innate immune system function is to maintain homeostasis and self- protection, it could also in certain circumstances result in autoimmunity.

2.11 Inflammatory mediators

Cytokines orchestrate the immune response. To maintain homeostasis a balance between pro- and anti-inflammatory cytokines is required. Proinflammatory cytokines such as (IFN- γ, TNF-α, IL- 12, and IL- 7) can play a role in the pathogenesis of MS. Different studies have shown elevated number of cells expressing TNF-α mRNA [180], serum TNF-α concentration [181], and PBMC secreting TNF-α [182] in MS patients. However, treatment with anti- TNF-α or soluble TNF-α receptor immunoglobulin fusion protein results to more frequent and prolonged relapses of MS. The data for IFN- γ in the blood of MS patients is also contradicting.

The data on antiinflammtory cytokines, such as IL- 4 and IL- 10, is also conflicting. IL-6 has both pro- and anti- inflammatory capacities. Raised levels of IL-6 have been seen in MS patient serum [182].

Chemokines and their receptors play a role in inflammation by recruiting leukocytes and other cell types. One important step is the trafficking of inflammatory T-lymphocytes into the CNS, which begins with weak adhesion and rolling on the endothelium of the BBB followed by firm arrest on the luminal side of the endothelium and subsequent diapedesis across the BBB.

Chemokines achieve this by the induction and activation of leukocyte adhesion molecules that mediate firm adhesion to the endothelium and establish a chemotactic concentration gradient that results in recruitment across the endothelial monolayer. The induction of proteolytic enzymes facilitates the breakage of the BBB [183], chemokines then mediate the retention of leukocytes in the CNS. Chemokine receptors role in the pathogenesis of MS have been widely studied. CC5 and CXCR3 have received attention as key receptors on Th1 cells, as have CCR3 and CCR4 on Th2 cells.

2.12 Infectious agents

In 1985 Sibley WA *et al* showed that MS relapses often followed viral infection [184]. The increase of incidence in small, previously isolated communities such as the Faroe Islands also direct the view towards an infectious agent [30].

Recent studies in EAE have focused on pathogens that can stimulate toll-like receptors. Toll-like receptors are highly conserved receptors that recognise pathogen-associated molecular patterns. Viruses that cause persistent infection in humans such as herpes and retroviruses have been widely studied as candidates for the contribution to the increased incidence of MS in some populations. Human herpes virus 6 (HHV-6) [185] and Epstein-Barr virus (EBV) [186 187] are the leading candidates. Among bacteria, Chlamydia pneumonia [188;189] has been implicated in MS.

2.12.1 Proposed mechanisms of infectious agent induction of MS

Two main mechanisms a) molecular mimicry and b) bystander activation have been proposed.

Molecular mimicry

This involves the activation of autoreactive cells such as T- and B- lymphocytes with peptides or antigenic determinants shared by infectious and self- antigens. The recognition of self- antigens at intermediate levels of affinity by T-lymphocytes during thymic selection leads to positive selection and export of these T-lymphocytes to the periphery. Cross reactivity between self- reactive T-lymphocytes with foreign antigens can lead to activation during infection, migration across the blood- brain barrier (BBB), CNS infiltration, and if they recognise antigens expressed in the brain, they cause tissue damage and potentially an autoimmune disease like MS.

Bystander activation

This phenomenon is classified into two categories:

- The activation of autoreactive T-lymphocytes by inflammatory cytokines, superantigens, and molecular pattern recognition, eg, Toll-like receptor (TLR) activation. This activation of T-lymphocyte is TCR-independent.
- Host antigens exposure as a result of viral damage and the effect of infectious agents on antigen- presenting cells (APCS). Virus-specific T-lymphocytes that are activated by the presence of virus migrate to the infected tissue, recognise viral epitopes and kill infected cells. This results in self-tissue damage and the release of autoantigen.

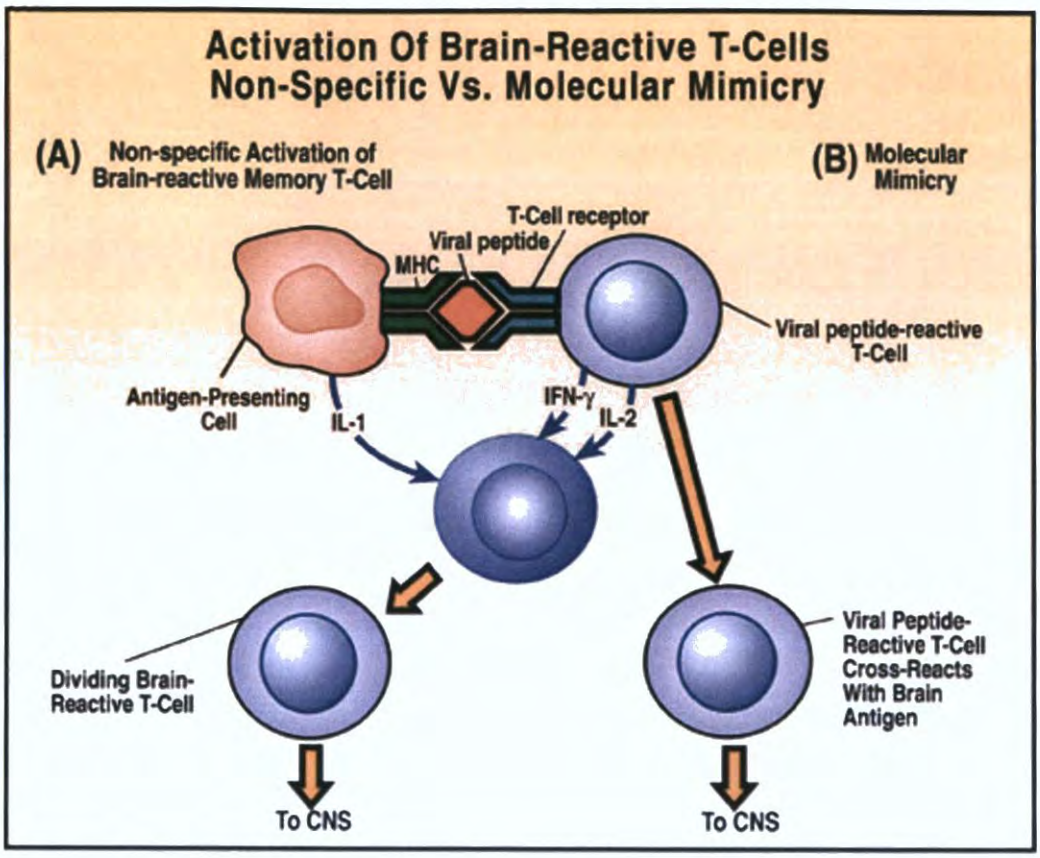


Figure 2.3. Possible aetiologic mechanisms of demyelination in CNS - T-lymphocyte

library.med.utah.edu/kw/ms/mml/ms_etiology01.html

2.13 Development of progressive disease

It is not entirely clear when and why progression occurs, but once established, irreversible progression appears to proceed at a steady rate in certain patients [190-192]. Studies suggest axonal degeneration could be the underlying cause for progression [193] thus once a threshold for the axonal injury is reached progression is initiated [194].

2.14 Neurodegeneration

In the past it was suggested that the neurological impairment seen in MS was solely due to demyelination. The view has since been changed and now the current view suggests the presence of neurodegeneration and axonal loss correlating better with the neurological disability seen in the disease [195]. Researchers suggest axonal damage occuring early in the disease secondary to demyelination [133 196] and neurodegeneration due to oligodendrocyte apoptosis has also been suggested to play a role in the formation of new lesions in MS [139]. Autoreactive T-lymphocytes are the likely drivers of these mechanisms.

2.15 Remyelination and repair

Recovery from relapses could be due to regulatory T-lymphocytes (Tregs), which are specialised T-lymphocytes with the ability to counter balance inflammation. It could also be due to the rearrangement of sodium channels [197].

Remyelination is the spontaneous repair process where new myelin sheaths are generated around demyelinated axons. 40% of MS lesions manifest some degree of remyelination [198]. It could result in partial restoration of the conductive properties of demyelinated axons [199 200]. The degree of remyelination depends on the mechanism and stage of progression of the lesion [201], although remyelination has been seen in patients dying at an old age [202]. It also depends on whether the environment within the plaque favours remyelination [203]. Also there is a positive correlation between remyelination and the number of surviving oligodendrocytes and a negative one with the presence of macrophages in the lesion [204].

2.16 Genetics of MS

2.16.1 Historical review of MS genetics

In 1868, Jean-Martin Charcot identified multiple sclerosis (MS) as a distinct neurological disease [4;112;205]. By 1870, American neurologists became more familiar with the "new" disease and new cases of MS were diagnosed. Shortly afterwards, MS became the focus of researchers and in 1896, Eichorst proposed that hereditary factors were involved in MS. In 1951, Pratt reviewed the tendency towards familial aggregation and suggested that more than one gene was required to explain the pattern of inheritance [205]. Since then, familial aggregation studies demonstrated an increased risk for MS among relatives of MS patients. A high concordance rate of 30% is seen in monozygotic twins and 2-5% in full siblings. For half siblings the percentage is only 1.1-1.4% but it is still considerably higher than in relatives by adoption in which the percentage is about 0.1% of the general population [206;207 208 209;210].

In the 1970's the first genetic association in MS was reported in the HLA region [211;212]. This was later specified to be HLA class II haplotype DRB1*1501,DRB*0101,DQA1*0102,DQB1*0602, often named DRB1*15 a split of DR2 [122 123].

Questions that cross the mind how significant are the genetic factors in the aetiology of MS, and how do they operate? Researchers nowadays believe that other genes may also be involved not only in the predisposition to MS but also to its different clinical phenotypes as well as patient's response to the current disease modifying agents. Over the past forty years and in spite of the

tremendous efforts that have been put into studying the genetics of MS, we still have not identified candidate genes that contribute to the pathogenesis of MS.

2.16.2 Major histocompatibilty complex (MHC)

Since several studies have been conducted demonstrating the importance of HLA class II in MS genetics, it is now recognised to be a genetic risk factor for MS. Class II molecules are known to be involved in antigen presentation to T lymphocytes, thus this association of HLA class II with the susceptibility to MS have also strengthened the hypothesis that MS is a T-lymphocyte mediated autoimmune disease. 60% of Western and Northern European MS patients carry the risk gene HLA-DR and DQ, HLA-DR15 haplotype (HLA-DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602 in comparison to only 30% of healthy controls. However, in Sardinia HLA DRB1*15 association is weaker occurring in 2.5% of MS patients and 1.5% of controls, it is mainly DRB1*03 and DRB1*04 that are associated with MS in this population [213-215].

The association of HLA-DR/DQ alleles with other genes, clinical course, MRI, or immunological characteristics of MS is not very clear. Examples of genes that are associated with DR15 haplotype are Transforming growth factor (TGF)-β family members, cytotoxic T-lymphocyte-associated antigen (CTLA)-4, the tumour necrosis factor (TNF) cluster, IL-1 receptor antagonist, IL-1, and oestrogen receptor [216-218]. Clinical factors associated with DR15 include earlier onset of the disease, higher incidence of RR MS, female gender, optic neuritis, or spinal involvement as initial event. Immunologically, higher CSF immunoglobulin, OCB, and matrix metalloproteinase 9 (MMP-9) levels have

been reported [219;220]. In addition, numerous attempts have been made to correlate between the DRB1*15 and the different clinical phenotypes of MS, as well as female gender and early onset of the disease, but no association with clinical outcome or disease severity has been established to date [221-223]. However, patients carrying DR4 allele often have worse clinical outcome, the disease is more progressive than patients carrying DR15.

In 2005, Dyment *et al* reported other class II alleles to be associated with MS: DRB1*17 was associated with increased risk of MS whereas DRB1*14 conferred protection to MS [224]. These findings suggest that additional alleles within the HLA class II locus may also influence the susceptibility to MS. Further work demonstrated an increased risk of developing MS by 6.7 fold in individual carrying two copies of the HLA DRB1*1501 allele compared to those carrying only one copy of the allele in which the risk increases by only 2.7 fold [225].

HLA-A, located in class I region, has recently been speculated to have an association with MS [226;227]. A large case/ control study suggested that HLA-A*0201 confers protection to MS [228].

Several mechanisms have been considered as to why are certain individuals carrying HLA class II alleles more susceptible to autoimmune disease including MS. Some of the mechanisms are: a) Preferential presentation of specific self peptides, eg, myelin peptides[229-231]. b) Reduced binding capacity causing incomplete thymic negative selection of self-reactive T-lymphocytes[232-234]. c)

Specific disease preventing interactions between polymorphic T-lymphocyte receptor (TCR) residue and α-helical regions of DR/DQ -α and -β chains[235;236]. d) Genes and protein expression of one or more disease associated DR and DQ alleles could be elevated in the CNS, enhancing antigen presentation[164;237]. e) Perturbed intracellular signalling events secondary to engagement of HLA class II molecules in patients with autoimmune diseases.

2.16.3 Linkage studies in MS

In the past decade, up to eleven microsatellite based whole genome linkage screens were carried out in a number of populations [238-241 215;242-247]. None of the above studies established concrete evidence for linkage. A meta-analysis was carried out by the GAMES and the Transatlantic Multiple Sclerosis Genetics Cooperative in 2003 included all microsatellite screens, showed evidence for linkage in the MHC, HLA-DRB1*15 region but no other linkage was confirmed in any other region. However, the study suggested linkage in two other chromosomal regions, 17q21 and 22q13 [248].

A single nucleotide polymorphism (SNP) based linkage screen was recently published in the American Journal of Human Genetics (AJHG) by the International Multiple Sclerosis Genetics Consortium (IMSGC)[249]. This involved DNA samples from 730 multiplex families in Australia, UK, USA and Scandinavia, a total of 2,692 individuals were genotyped for 4,506 SNPs. The analysis showed evidence for linkage in the MHC region with LOD 11.7 in chromosomal regions 19q13, 17q23 and 5q33. Also genome wide screens carried out in multigenerational families with several affected relatives showed

no other regions to be significant apart from those for linkage on chromosome 9q and 12p12 [250 251;252].

2.16.4 Association studies in MS

Over the past decade, a number of studies were conducted searching for non-HLA candidate genes but again the results have been inconclusive [14;253 217;254-256]. Candidate genes were selected on the bases of their biological function mainly their role in inflammation and neurodegeneration also their chromosomal location with presumed importance in autoimmune diseases. The lack of success in the search for candidate genes could be attributed to the heterogeneity of the disease.

The first genome wide association (GWA) study was recently published in the New England Journal of Medicine, a total of 12,360 individuals were included in the analysis. IL2RA and IL7R are the two genes identified presenting significant association with MS [257].

2.17 Other Risk-Conferring Genes

In 2004, Dyment et al published a review article on the genetics of MS, in which he pointed out the search for candidate genes in MS has been plagued by initial positive results followed by negative, inconclusive results[217]. Examples of such are polymorphisms of TCR genes, immunoglobulin loci, CCR5, and CD45. Few loci have been identified including TCR- β chain locus, CTLA-4, TNF- α and- β alleles, and ICAM-1. CCR2, IL-10 receptor α, FAS-L may confer protective effects; CCR5, IL-10, IL-4 receptor α, IL-2 receptor β, IFN-γ, vitamin D, and

oestrogen receptor confer risk. Other genes that are implicated in the disease process are Notch4, a transcription factor involved both myelin development and immune function, neutral sphingomyelinase activating factor, ciliary neurotrophic factor, and the myelin basic protein (MBP) gene. Apolipoprotein E (APOE4), which is involved in lipid metabolism and associated with the severity of Alzheimer's disease, shows an association with a faster and more severe progression of MS, although its significance in MS remains controversial.

Other loci have been found to be of importance, in particular, loci on the short arms of chromosomes 2, 3, 5 and 7 and on the long arms of chromosomes 2, 17 and 19 are likely to contain relevant genes. Existing data that has been focused on candidate genes chosen on the grounds of the hypothesis about MS pathology have been controversial. The T-lymphocyte-receptor α- and β-chain genes and the immunoglobulin genes have been greatly studied. More recently studied gene is the gene encoding CTLA-4 which may influence the susceptibility to MS and other genes, in particular the interleukin-1 cluster, may influence the course of the disease.

Chapter 3

Materials and Methods

This chapter describes, in details, the different methods and techniques used to study T lymphocyte gene expression profile in MS.

3.1 *In silico* data mining

3.1.1 Digital differential display (DDD)

DDD is a computer-based technology developed at National Centre of Biotechnology Information (NCBI) that utilizes and harnesses Expressed Sequence Tags (EST) expression data found in UniGene libraries. It is a tool that facilitates the comparison of EST's abundance in selected tissue libraries and thus provides a quantitative measure of gene expression. The final output is analysed using the Fisher Exact test to detect statistically significance differences ($P \leq 0.05$).

3.1.2 Definitions of terms used for the *in silico* data mining

- UniGene is a gene discovery resource. It is an automated system for producing an organised view of the transcriptome. It divides GenBank sequences, including EST's, into a non- redundant set of gene- oriented clusters. Each UniGene cluster contains sequences of well- characterised genes as well as novel EST sequences that represent a single gene. The database is updated weekly.

- GenBank is a DNA sequence database, to which data is submitted by the international scientific community. This data is accessible through NCBI website.
- Expressed sequence tags (EST) represent a snapshot of expressed genes in a given tissue and/or at a given developmental stage. It is a short strand of cDNA sequence (usually 200-800 nucleotides long), representing a portion of an entire expressed gene. EST's are assembled from messenger RNA (mRNA) sequences that represent copies from expressed genes. Since RNA cannot be cloned directly, it is reverse transcribed to single-stranded cDNA using the enzyme, reverse transcriptase. The newly synthesized cDNA is cloned to generate EST libraries. EST's are submitted to GenBank and database of EST's (dbEST) as batches of entries.

3.1.3 Digital extractor (DE)

Digital Extractor is a program used for the high throughput processing of data set derived from DDD based comparison of EST libraries [258]. It integrates and utilizes a number of tools including: a) CAP3 [259], an EST assembly program to produce contiguous sequences; b) RepeatMasker [260], for masking repetitive elements within the assembled EST contigs; and c) BLAST [261], for homology searching within UniGene clusters.

3.1.4 *In silico* identification of genes using DDD

In this study, we compared brain tissue libraries of MS patients versus libraries of normal adult human brain tissue to identify genes that are differentially

expressed in MS. After selection of the libraries for comparison, the result is delivered in a table (Figure 3.2).

3.1.5 Annotating EST's using DE

A major limitation of *in silico* gene mining approaches is the cumbersome nature of the subsequent data analysis. The output from DDD is a list of UniGene clusters representing known genes, and sequences without homology to known genes that were significantly altered between selected tissue libraries. To expedite this strategy the data derived from the DDD comparisons were processed using Digital Extractor. This application provides for high throughput processing of DDD output, by performing automated annotation of the output clusters. Digital Extractor was used to compile the profiles of known genes differentially expressed and also to annotate those clusters containing cDNAs without homology to known genes. It utilises CAP3 (a DNA sequence assembly program) for assembly of EST clusters, RepeatMasker to mask repetitive elements and BLAST (basic local alignment search tool) for gene identification [258]. Having completed the annotation of the dataset ontological classifications of the differentially expressed genes was completed using Onto-Express.

On the following page a schemtic representation of the DDD is illustrated in Figure 3.1

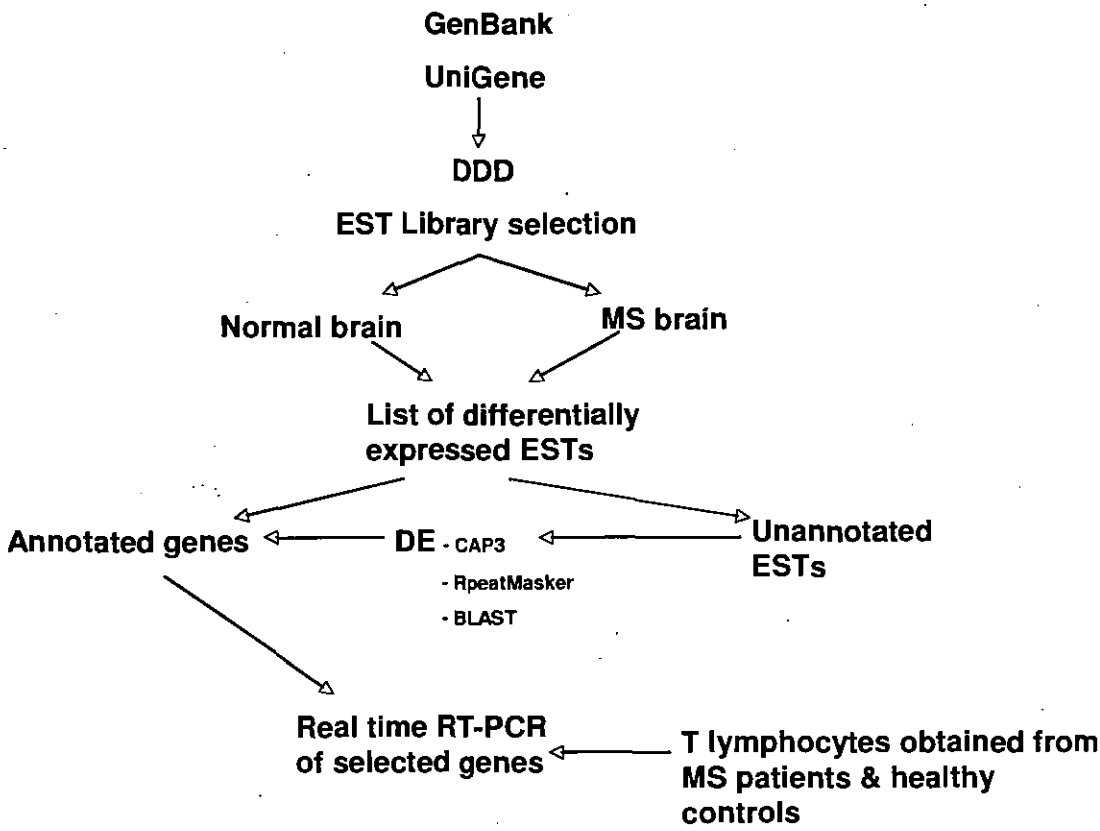
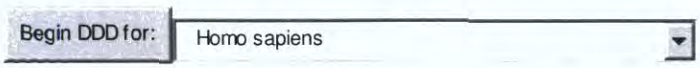


Figure 3.1 Schematic Presentation of the DDD strategy

Figure 3.2. Digital Differential Display (DDD) output format

A: normal brain, B: MS brain. Numbers represent frequency of the transcript (eg. 278) and results are sorted by significance. Hs- UniGene number



For the current analysis, the following table describes the pools that have been defined so far.

	Pool	Library ID(s)	Clustered ESTs
Edit...	**A.** Normal adult brain	6309, 959, 314, 6000, 6001, 128	91576
Edit...	**B.** MS brain	205	11516

New...

Start Over

Statistically Significant Differences

A normal..	B ms b	Gene index	Gene description
278 0.00304	0 0.00000	Hs.84389	synaptosomal-associated protein, 25Kd (SNAP25)
A>B	B<A		
265 0.00289	0 0.00000	Hs.115352	growth hormone 1 (GH1)
A>B	B<A		
237 0.00259	17 0.00185	Hs.1905	prolactin (PRL)
A>B	B<A		
178 0.00194	1 0.00009	Hs.82749	transmembrane 4 superfamily member 2 (TM4SF2)
A>B	B<A		

3.2 Laboratory work

3.2.1 Lymphocyte isolation

Fifty millilitres of fresh blood was collected into EDTA blood bottles from patients and controls by venepuncture. Blood from each sample was divided equally into two 25ml portions and put into 50ml sterile tubes. 15ml HBSS (Gibco-Hank's Balanced Salts, Biosciences, Dublin, Ireland) was added to each tube. 10ml of Ficol (Amersham Biosciences UK Ltd, Bucks, UK) was put into two separate fresh 50mls sterile tubes, then the 40ml mix of each blood and HBSS bottle was carefully layered on top of the Ficol. This was centrifuged at 12,000 RPM for 25 minutes at room temperature. The buffy coat was aspirated using a pipette, centrifuged at12,000 RPM for 10 minutes at room temperature then the newly formed pellet was washed with PBS (Phosphate buffer salt) and centrifuged at 12,000 RPM for 10 minutes at room temperature twice. The supernatant was discarded and the remaining pellet was resuspended in E-lyse (Human erythrocyte lysing kit, R&D Systems Europe, Oxon, UK) left for 10 minutes, then 45ml of wash buffer was added and the mix was centrifuged at 12,000 RPM for 10 minutes at room temperature. Again the supernatant was discarded and the pellet obtained was resuspeded in 1ml column buffer (Human T-lymphocytes enrichment columns, R&D Systems Europe, Oxon, UK). Simultaneously, two Human T-lymphocytes enrichment columns were placed on a column rack (R&D Systems Europe, Oxon, UK) and a 15ml sterile tube was placed under them. The columns were washed three times with 2ml column buffer solution (Human T-lymphocytes enrichment columns, R&D Systems Europe), the collected solution at the bottom of the columns was then discarded. Fresh 15ml sterile tubes were placed at the bottom of the columns. Next, 1 ml of

the dissolved pellet was added onto the washed columns, left for few minutes and collected at the bottom of the columns into 15ml sterile tubes which were placed at the bottom of the columns, the columns then were further washed five times 2ml at a time with the column buffer (Human T-lymphocytes enrichment columns, R&D Systems Europe). The collected sample was centrifuged at 12,000 for 10 minutes at room temperature. The supernatant was then discarded and the pellet obtained was resuspended in 1ml of Trizol (Invitrogen Life Technologie Ltd., Paisley, UK) in 1.5 ml sterile eppendorf and stored at -80°C for further work.

3.2.2 Generation of MBP-specific T-lymphocyte lines

The buffy coat was collected, following the first few steps described above, from fresh blood taken from healthy individuals. Myelin basic protein (MBP)-specific T-lymphocyte lines were generated using a modified split-well technique [262].

PBMC's were isolated from freshly collected blood from Healthy individuals using ficoll-hypaque gradient centrifugation. The cells (2 X 10^5 /well) were seeded in complete medium (CM; consisting of RPMI 1640 supplemented with 5% human AB serum, 2Mm L-glutamine, and 100 U/ml penicillin-streptomycin) (Sigma- Aldrich Ireland Ltd) into 4 X 96 well plates. Two of the plates were stimulated with 10 µg/ml of human MBP (Sigma- Aldrich Ireland Ltd). Seven days later, 10 U/ml IL-2 (Sigma- Aldrich Ireland Ltd) was added to each well of one of the unstimulated plates and one of the stimulated plates.

After another seven days, the cells were collected, centrifuged at 3,000 RPM for 7 minutes at room temperature.

3.2.3 Cell counting

Cultures showed positive response to MBP in the split-well, the cells clumped together. Using low power microscopy the cells that were stimulated with MBP showed clumping. Cell counts were executed using a Neubauer haemocytometer slide. Cell viability was assessed using the trypan blue 0.4% (SIGMA) exclusion dye technique. Twenty microlitres of trypan blue was added to 100 µl of cell suspension, vortexed, and left to incubate for 2 minutes at room temperature. After adding 20 µl of the mixture to the haemocytometer counting chamber the cells were visualised by light microscopy. Viable cells exclude the dye and appear clear while dead cells cannot and are stained blue. The four outer quadrants are counted and an average calculated.

3.2.4 Isolation of total RNA from cells

The following method was used to both isolate RNA from patient's lymphocytes and also the cultured cells. Total RNA was harvested using TRIZOL reagent (SIGMA, United Kingdom) as previously described [263]. When cells were 80% confluent RNA was isolated. Growth media was removed and 1ml of TRI-Reagent® was added per 75cm^2 flask and left at room temperature for 5 minutes with occasional agitation. Once microscopy confirmed cell lysis, the suspension was placed in1.5 ml sterile eppendorf. Two hundred microlitres of chloroform was added to the mixture, vortexed for 10 seconds, and then left at room temperature for 15min followed by centrifugation at 12,000 x g at 4°C

for 15min. Taking care not to disturb the DNA containing lower and protein containing interphase layers the upper aqueous layer was transferred to a fresh 1.5ml microfuge tube and 0.5 ml isopropanol was added. After mixing the sample by gently inverting the tube repetitively, the tube was left to stand at room temperature for 10min followed by centrifugation at 12,000 x g at 4°C for 10min. The supernatant was removed and the pellet was washed by gentle vortexing with 1ml of sterile 75% ethanol. The tube was centrifuged at 7500 x g for 5min to collect the pellet. After the removal of the ethanol without disturbing the pellet, the open cap eppendorf was allowed to air-dry for 5min. 50µl of 0.1% DEPC treated H_2O was used to resuspend the pellet using heated rack at 60°C for 15min and the resuspended RNA was store at –80°C.

3.2.5 RNA quantitation and analysis

UV Spectrophotometry was performed on total RNA at a 1/100 dilution (5µl in 500µl 0.1% DEPC treated H_2O) using a Pharmacia Biotech Ultrospec 3000 spectrophotometer and a quarts cuvette. The absorbance values at 260(A_{260}) and 280(A_{280}) nm were recorded. To calculate RNA concentration of each sample we used the formula: (A260) x 40 (RNA extinction coefficient) x dilution factor (i.e. 500/5) x final sample volume = RNA yield in µg. The A_{260}/A_{280} ratio gave an indication of RNA purity where a value of 1.8 was acceptable; a value greater than 1.8 indicted protein contamination, and a lower value suggested organic solvent contaminants.

The integrity of RNA was assessed using denaturing agarose gel electrophoresis. A 1% agarose gel was prepared by boiling agarose in 1X Tris Acetate EDTA (TAE) (40 mM Tris-acetate, 1 mM EDTA, pH 7.6) obtained from Gibco Life Technologies (Paisley, Scotland). After allowing the gel to cool the gel was cast in a BioRad sub-cell electrophoresis system. 1 µl RNA loading buffer (62.5% (v/v) deionised formamide; 1.14 M formaldehyde; 200 µg/ml Bromophenol blue; 200 µg/ml xylene cyanole; 1.25 X MOPS-EDTA-sodium acetate; 50 mg/ml ethidium bromide) was mixed with 2µl RNA and heated at 65°C for 10min then cooled on ice prior to loading on the gel. The running gel was composed of 1 X TAE buffer and was run at 5V/cm for 45min or until the dye front had run sufficiently along the gel. The gel was then visualised on a UV transilluminator, with the presence of two and sometimes three stained bands representing the 28S, 18S and 6S subunits indicating intact RNA (Figure 3.3). A smear along the length of the gel represents a degraded sample of RNA. Integrity of the RNA was also confirmed using Agilent Bioanalyzer RNA 6000nano Chip Assay (Figure 3.4).

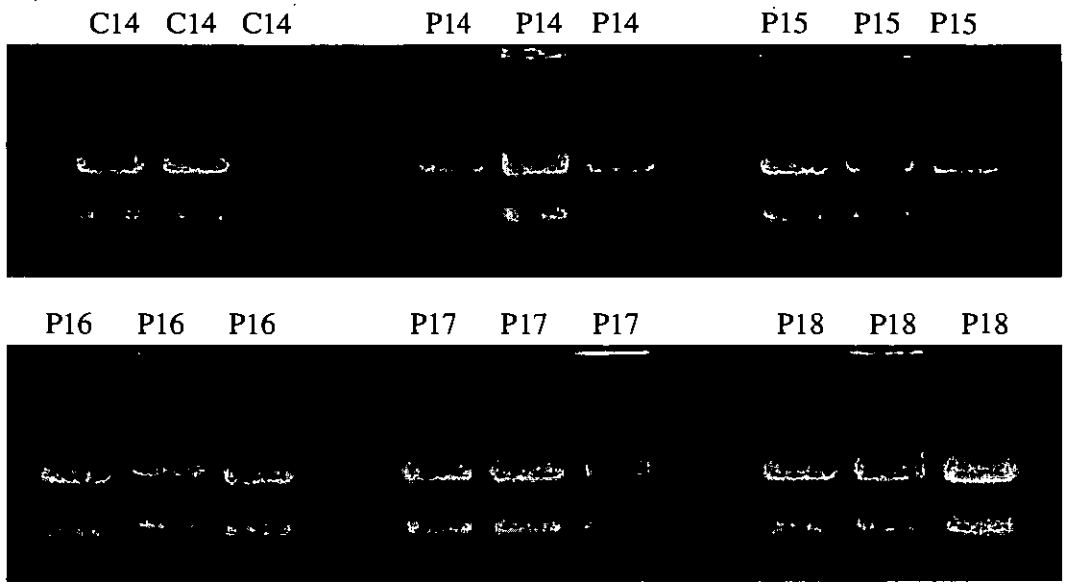


Figure 3.3. This figure shows an RNA gel derived from lymphocytes from the MS patient cohort and controls performed in triplicate. C =control, P= patient)

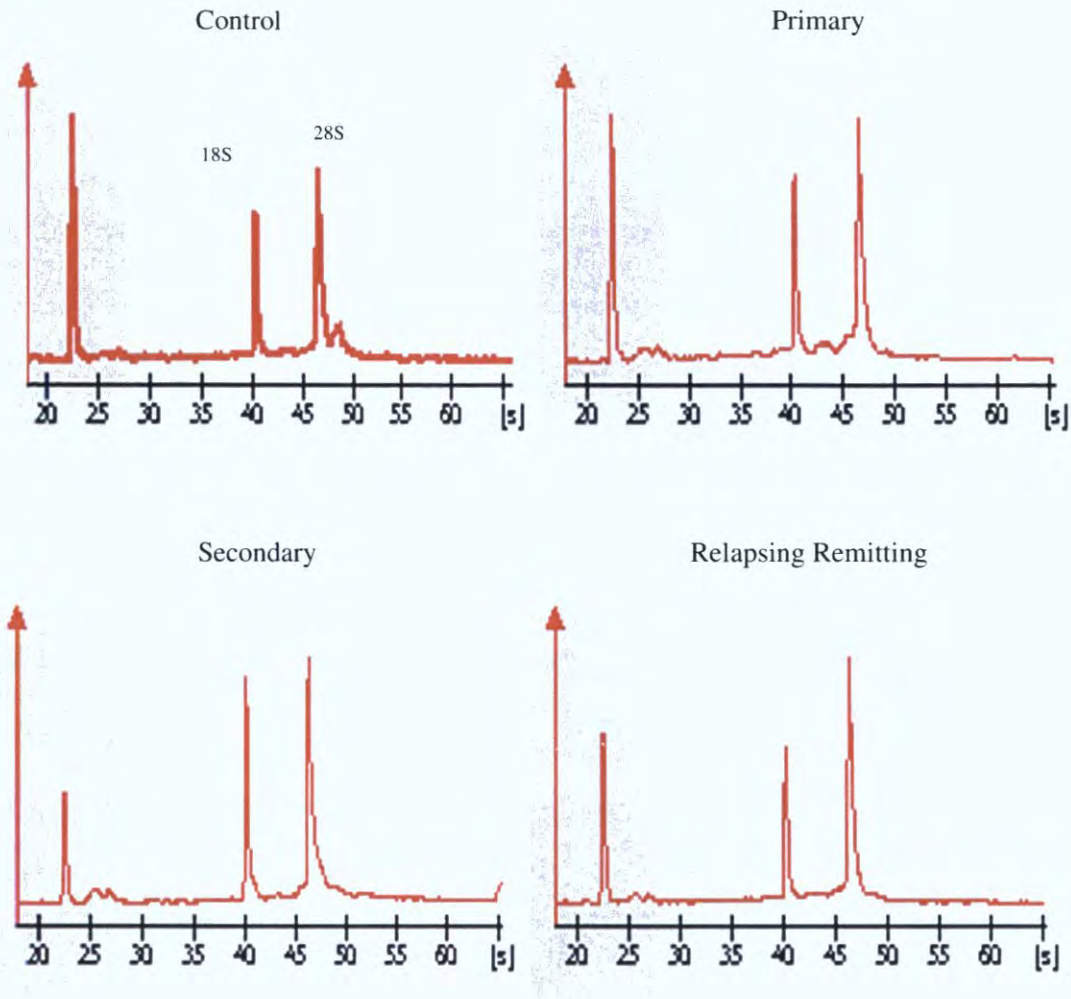


Figure 3.4. Representative figure of results from Agilent 2100 bioanalyser testing performed on RNA extracted from lymphocytes from MS patient cohort and controls

3.2.6 DNA isolation

10 ml of whole blood was collected from 11 adults using aseptic technique. DNA was extracted using QIAamp DNA Blood Midi Kit (Qiagen Ltd, West Sussex, UK) as follows. 2ml of frozen whole blood from each sample was thawed at 37°C with regular mild agitation and stored on ice before commencing the procedure. 200µl of QIAGEN Protease was pippetted into the bottom of 11 separately labelled 15ml centrifuge tube (one tube per sample) then 2ml whole blood from each sample was then added to each tube labelled for the sample and mixed briefly. To that mixture 2.4ml Buffer AL was added to each bottle and mixed by inverting the tube 15 times followed by vigorous shaking for at least one minute then the tubes were incubated at 70°C for 12 minutes. 2ml Ethanol (96-100%) was then added to the sample and mixed by inverting the tubes 10 times followed by additional vigorous shaking. Half of the solution was then transferred into QIAamp Midi columns placed in 15ml centrifuge tubes (provided by kit), taking care not to moisten the rim. Cap was closed the columns were centrifuged at 3,000 rpm for 3 minutes. QIAamp Midi columns were then removed from the centrifuge, filtrate was discarded and QIAamp Midi columns were placed back in the15ml centrifuge tubes and the remainder of the solution from the previous step was transferred into QIAamp Midi columns (same procedure was repeated and filtrate was again discarded). 2ml Buffer AW1 was then added to the QIAamp Midi columns, cap was closed and columns centrifuged at 5,000rpm for one minute (this time filtrate was not discarded). 2ml Buffer AW2 was added to QIAamp Midi columns, cap closed and centrifuged at 5,000rpm for 15 minutes. Then the QIAamp Midi columns were placed in 15ml centrifuge tubes (provided by kit), this time the used

collecting tubes were discarded with the filtrate. 300µl Buffer AE at room temperature was directly added onto the membrane of the QIAamp Midi columns, cap was closed. The columns were incubated at room temperature for 5 minutes and then centrifuged at 5,000rpm for 2 minutes. For a maximum DNA yield the final step was repeated again. The filtrate were then stored in 1.5 ml eppendorf and stored at -80°C for later use.

3.2.7 Reverse transcription

The following steps were used to prepare cDNA. From each sample 1 µg of RNA was pipetted into a 0.5 ml PCR sterile tubes. The RNA was treated with 10X DNase I (Invitrogen, Life Technologies, Paisely, United Kingdom), left for 15 minutes at room temperature to digest the single and double strands before cDNA preparation. To stop the reaction 1µl of 25 Mm EDTA was added to inactivate DNase I, this was followed by heat inactivation by heating the sample to 65 °C for 10 minutes. To make cDNA the following master mix was prepared and added to the mixture: 5X First strand Buffer, random primers, dNTP's, 0.1M DTT and Superscript™ II RNase H⁻ reverse transcriptase, all obtained from Invitrogen, Life Technologies, Paisely, United Kingdom. The reaction mixture was then incubated at 25 °C for 10 minutes (primers); 42 °C for 50 minutes (reverse transcription), and 70 °C for 15 minutes (enzyme inactivation). The samples were then stored at -20 °C.

3.2.8 Real time quantitative PCR

The Taqman Universal PCR Master Mix Kit (Applied Biosystems) was optimized for TaqMan reactions and contain AmpliTaq Gold DNA Polymerase,

AmpErase UNG, dNTP's with dUTP, passive reference dye (ROX), and optimized buffer components. The following genes were studied:

Annexin A1 Hs.00167549

Zinc-fingers and homeoboxes 1 Hs.00232545

Apolipoprotein E Hs.00171168

Prion protein Hs.00382657

SAM domain and HD domain 1 Hs.580681

Cytotoxic T-lymphocyte-associated protein 4 Hs.00175480

Interferon regulatory factor 1 Hs.00233698

Major histocompatibility complex, class II, DR alpha Hs.00219575

Interferon regulatory factor 4 Hs.00180031

The default thermal conditions for the PCR amplification were as follows: 2 minutes at 50 °C to activate AmpErase UNG, 10 minutes at 95 °C to activate AmpliTaq Gold Enzyme, 15 seconds at 95 °C to denature DNA, and 1 minute at 60 °C to anneal and extend template. The denaturation and extension steps ran for 40 cycles following the initial enzyme activation. To ensure specificity a melt curve analysis was carried out starting at 50°C and ramping to 90°C. One peak in the melt curve indicates no secondary, non-specific products were formed during the reaction. Relative gene expression levels were calculated using ΔCT method with 18S as a control.

3.3 Microarray expression profiling

RNA isolation, cDNA synthesis, *in vitro* transcription and microarray analysis were performed as previously reported [264].

3.3.1 cRNA synthesis and labeling

First and second strand cDNA were synthesized from 5-15 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Gibco Life Technologies) and oligo-dT_{24}-T7 (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-3') primer according to the manufacturer's instructions. cRNA was synthesized labeled with biotinylated UTP and CTP by in vitro transcription using the T7 promoter coupled double stranded cDNA as template and the T7 RNA Transcript Labeling Kit (ENZO Diagnostics Inc.).

Briefly, double stranded cDNA synthesized from the previous steps were washed twice with 70% ethanol and resuspended in 22 μl Rnase-free H_2O. The cDNA was incubated with 4 μl of 10X each Reaction Buffer, Biotin Labeled Ribonucleotides, DTT, Rnase Inhibitor Mix and 2 μl 20X T7 RNA Polymerase for 5 hr at 37°C. The labeled cRNA was separated from unincorporated ribonucleotides by passing through a CHROMA SPIN-100 column (Clontech) and precipitated at –20°C for 1 hr to overnight.

3.3.2 Oligonucleotide array hybridization and analysis

The cRNA pellet was resuspended in 10 μl RNAase-free H_2O and 10.0 μg was fragmented by heat and ion-mediated hydrolysis at 95°C for 35 mins in 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc. The fragmented cRNA was hybridized for 16hr at 45°C to HGU133A Plus 2.0 oligonucleotide arrays (Affymetrix) containing ~32,000 full length annotated genes together with additional probe sets designed to represent EST sequences. Arrays were washed

at 25°C with 6 X SSPE (0.9M NaCl, 60 mMNaH2PO4, 6 mM EDTA + 0.01% Tween 20) followed by a stringent wash at 50°C with 100 mM MES, 0.1M [Na+], 0.01% Tween 20. Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to constant target intensity (250) for all arrays used.

3.3.3 Introduction to microarray chip analysis

The analysis of oligonucleotide microarray data involves a number of steps (Figure 3.5). Once the initial experiment is complete, the RNA hybridised to the chip and an image is produced. The data produced is normalised and the expression indices analysed. The method of analysis utilises cluster analysis where genes with similar expression profile are grouped together. In clustering the data consist only of the gene expression values. The analytical goal is to find clusters of samples of genes such as that observations within cluster are more similar to each other than they are to observations in different clusters. Cluster analysis can be viewed as a data reduction method in that the observations in a cluster can be represented by an 'average' of the observations in that cluster. This method presumes that if a number of genes cluster then these genes most likely are involved in similar functions.

Comparison analysis may also be used. In order to detect and quantify changes in gene expression in two samples hybridized to two genechip arrays of the same type comparison analysis is performed. One of the arrays is designated; the baseline and the other array; the experiment. The difference value perfect match and mismatch (PM-MM) of every probe pair in the array-designated

baseline is compared to its matching probe pair on the array designated experiment. Two sets of algorithms calculate change difference and change quantity metrics for each probe set. The output from these algorithms is in the form of a change p-value, and a quatitative assessment of change in gene expression in the form of Signal Log Ratio.

Principle component analysis (PCA) is also used for microarray analysis through examination of the variance in the dataset. What PCA attempts to do is reduce the dimensionality of the data and summarize the most significant parts while filtering out noise. In this way it identifies patterns in the data and expresses them in a way that illustrates their similarities and differences. PCA can be used as data set to capture the cluster structure prior to cluster analysis.

Using these methods of analysis differential gene expression among the experiment conditions can be identified. Genes of interest can then be identified and their putative function tested.

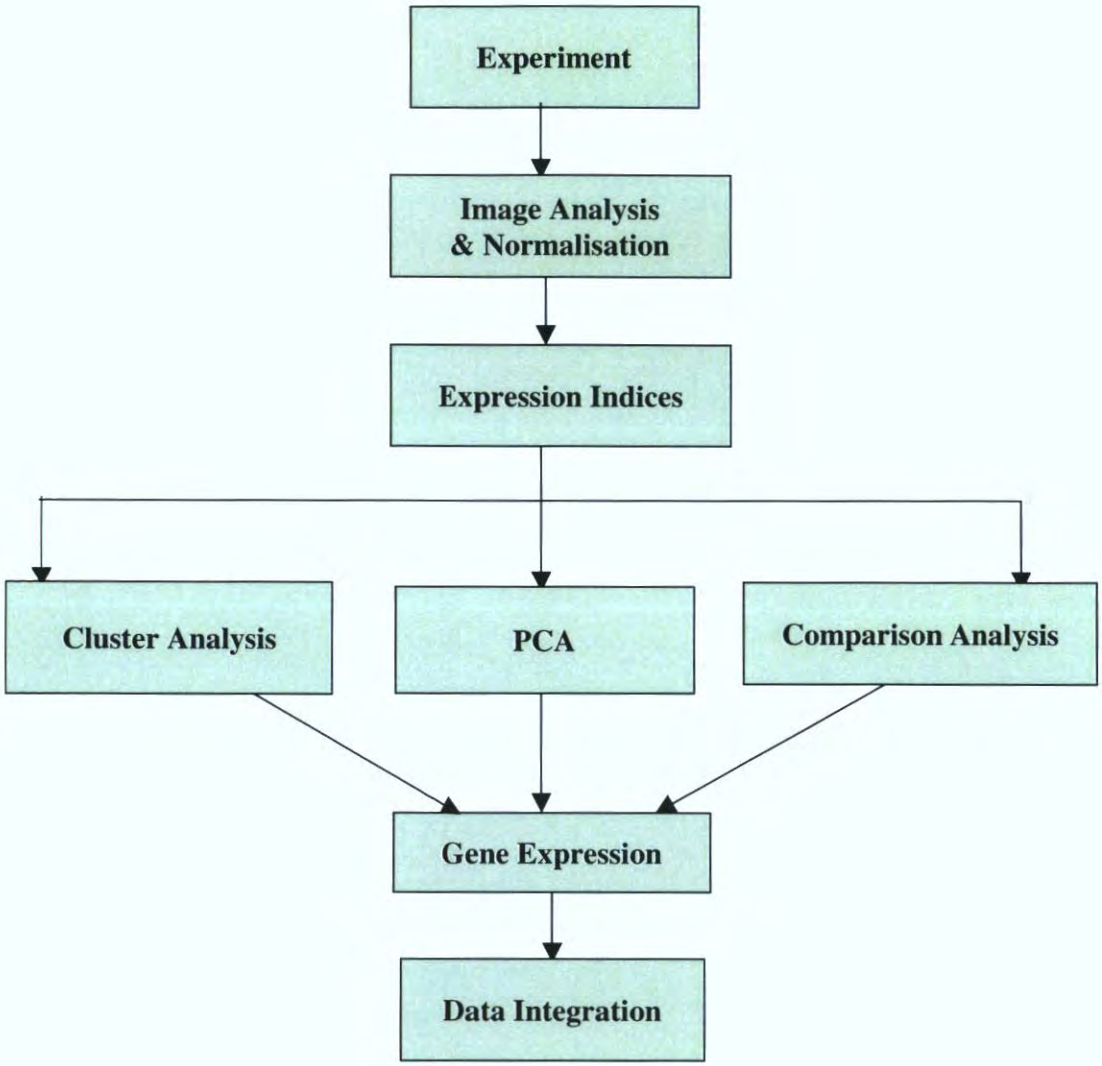


Figure 3.5. Schematic of microrray data analysis

3.3.4 Normalisation of array data

Normalisation of the data was achieved by using Robust Multichip Analysis (RMA). RMA is an R (a language for statistical computing and graphics) based technique that analyses data directly from the Affymetrix microarray *cel* image file. The analysis involves three operations; background adjustment, quantile normalisation and summarisation [265](Figure 3.5). Firstly, probe-specific background correction is performed to compensate for nonspecific binding by using PM distribution rather than PM-MM values; secondly, probe-level multichip quantile normalization is performed to unify PM distributions across all chips; and lastly, it executes a robust probe-set summary of the log-normalized probe-level data by median polishing[265]. In order to use a Microsoft Windows operating system RMAexpress was used. This package is a stand-alone GUI programme specifically designed to operate on Microsoft Windows and exclusively performs RMA.

To ensure robust data analysis following normalisation, those genes with very low expression at all time-points were filtered out. A signal log intensity (SLI) of greater than 5.0 was used as cut off. In addition, an average RMA value was computed for each category to facilitate further bioinformatic analysis. To ensure that the mean was statistically representative of each individual array a standard error of the mean was generated and a SEM ≤ 0.5 was taken to imply sufficient similarity between arrays for inclusion in further analysis. Thereafter, expression data for disease category was compared to control and a mean signal log ratio of 0.6 or greater (equivalent to a fold change in expression of 1.5 or greater) was taken to identify significant differential regulation.

As each data from patients with different MS clinical phenotype was microarrayed in duplicate, an average RMA value was computed. To ensure the average was statistically representative a t-test and p-value were generated. Only those genes with a p-value of ≤ 0.01 were included in subsequent bioinformatic analysis.

3.3.5 Microarray chip analysis

Using normalised RMA values, cluster analysis was performed by Eisen's programme of Unsupervised Average Linkage Hierarchical Cluster Analysis [266]. In cluster analysis genes with comparable patterns of expression are grouped together by employing mathematical methods of similarly organized patterns of expression. Initially all the genes under study are assessed and the two closest genes are joined creating the first node. Subsequent nodes are determined and added by the pair wise joining of genes, based on the distance between them, culminating in all genes belong to the one node. To ensure tight cluster analysis and reduce the background noise that accompanies microarray experiments the 32,000 genes of the Affymetrix HGUA133A Plus 2.0 chip were filtered to remove genes with very low expression for all patients. An initial cluster analysis of each individual array and the average array for each patient was performed to ensure that each patient were sufficiently similar in expression profiles to permit the use of average values thereafter. A list of genes was created via the publicly available DAVID 2.0 databases.

3.4 DNA processing using Affymetrix SNP chip

3.4.1 HLA typing, Karyotyping and GeneChip

DNA isolated from whole blood was used to determine the HLA typing of the family (HLA-DR and DQ) (Tissue typing, Department of immunology, Beaumont Hospital, Dublin 9). The method used to type for HLA-DRB1* and HLA-DQB1* was PCR-SSO. The principle procedure is based on PCR target amplification, hybridisation of amplified products to an array of immobilized sequence-specific oligonucleotide probes, and detection of the probe-bound amplified product by colour formation. A negative contamination control and two known positive DNA controls were used to validate the sample run. Karyotyping was performed for one affected individual (National Centre for Medical Genetics, Our Lady's Hospital for Sick Children, Crumlin, Dublin 12). Also the Affymetrix GeneChip Human Mapping 250K Nsp Array (Aros Applied Biotechnology A/S, Arhus, Denmark) was used to produce genome-wide SNP genotypes for 12 members of the family. Two hundred and fifty nanogram Genomic DNA was used as starting material for the Affymetrix Mapping 250K Nsp Assay. The 250K assay was done according to the manufacturer's protocol www.affymetrix.com. Ninety micrograms of PCR product were fragmented, labelled and hybridised to the 250K array. SNP calls were generated using the Affymetrix GeneChip Genotyping Analysis Software version 4.0. A P value cut off threshold of 0.33 was used for the data analysis.

Genotypes for the 262,264 Single Nucleotide Polymorphisms (SNPs) were cleaned and the data set was greatly reduced in size by screening for the most informative markers on the basis of minor allele frequency greater than 1%. The

father's genotypes showed a high drop-out rate compared with the other pedigree members, leading to his exclusion in order to maintain data quality. Inter-marker linkage disequilibrium was reduced to a minimum by removing one marker of any pair of markers showing an r^2 value less than or equal to 0.05 in order to reduce spurious inflation of allele sharing [267]. Due to the small number of founders genotyped, allele frequency and inter-marker LD data was taken from the HapMap genotyped CEU individuals (www.hapmap.org). SNPs in the pseudoautosomal region (PAR1) of the X chromosome were excluded due to methodological difficulties with analysing male heterozygous markers [268]. A total of 38,841 SNPs were retained for analysis.

3.4.2 Statistical and data analysis

A multi-point non-parametric linkage statistic was calculated, and traditional parametric linkage analysis was conducted under two inheritance models: a recessive model and a model of partial dominance, where penetrance was set at 0.4 for disease allele carriers. The phenocopy rate was set close to the population prevalence of 0.0015, and disease allele frequency was assumed to be 0.001. MERLIN software was used for all linkage analysis [269]. The accompanying MINX program was applied to the X chromosome markers, excluding PAR1. Statistical significance levels were established empirically by simulating genotypes (gene-dropping) and repeating the analysis over 100 (non-parametric) or 200 (parametric analyses) replicates [270]. A locus-counting approach was also investigated to determine if the number of peaks with at least a particular LOD score threshold value, exceeded the number expected by chance [271].

3.5 Statistics

Statistical analysis for the real time RT-PCR data was carried out using ANOVA one-way analysis of variance with Student-Newman correction. Significance was assumed for values of $p= 0.05$.

Chapter 4

In silico UniGene libraries used to identify possible T-lymphocyte specific gene expression in MS lesions

4.1 Aim

To identify a cohort of genes that found to be differentially expressed in MS brain tissue and are likely to be of importance in T lymphocytes activation in MS. We aim to achieve this by using an *In silico* data mining tool using expressed sequence tags (EST) library comparisons of normal brain tissue and MS brain tissue. The cohort of genes will then be examined in T-lymphocytes obtained from MS patients with different disease phenotypes and healthy controls. This may lead to the identification of markers of MS phenotypes.

4.2 Hypothesis

We propose that changes in T-lymphocyte gene expression profiles drive the initiation and progression of disease in MS. Additionally, particular MS phenotypes may be associated with differential gene expression in T-lymphocytes.

4.3 Objective

Here, using publicly available MS brain tissue data we aim 1) To identify a cohort of genes dysregulated in MS brain that have putative roles in activated T-lymphocytes. 2) To examine the expression of identified genes in peripheral T-lymphocytes of MS patients as part of an ultimate goal of further defining the role of the T-lymphocyte in the pathogenesis of MS.

4.4 Background and rationale

Recent advances in molecular biology have had an influence on how scientific research is conducted. New internet based resources have become of considerable help to research: the National Centre of Biotechnology Information (NCBI) is a publicly available website whose databases and software provides an extensive reservoir of knowledge for the research and medical community (http://www.ncbi.nlm.nih.gov/sites/entrez). Digital Differential Display (DDD) which is contained within NCBI is a computational method for comparing the expression profiles of nucleotide sequences across individual cDNA libraries or pools of libraries and as such can be used for the rapid identification of novel genes, disease markers and potential therapeutic targets in many complex disease [272 273](http://www.ncbi.nlm.nih.gov/UniGene /ddd.cgi). In MS, where access to brain tissue is limited, DDD has the potential to be a very useful technique. Through DDD, RNA sequences from normal and diseased brain tissue can be compared.

MS is a demyelinating disease of the central nervous system (CNS) white matter resulting in plaque formation. Histologically, plaques are found in periventricular areas and often surround one or more blood vessels. Also found within the plaque is an inflammatory infiltrate which mostly comprises of T-lymphocytes and macrophages [17;274;275]. Considering the scarcity of brain tissue available to researchers, DDD offers a unique technology to explore gene expression in MS lesions.

T-lymphocytes play a pivotal role in the pathogenesis of MS [116 119]. As mentioned above, a key component of the MS lesions are T-lymphocytes and these are found in all four histological subtypes [140]. Additionally, there is experimental evidence to demonstrate the presence of primed T-lymphocyte in the peripheral blood and cerebrospinal fluid (CSF) of MS patients [276 277]. More specifically, these peripheral T-lymphocytes appear to be activated myelin-reactive T-lymphocytes [278]. Experimental work proposes that the peripheral T-lymphocyte becomes activated and migrates to the CNS where it causes an inflammatory response that result in demyelination. In turn, demyelination results in release of cytokines and other factors such as myelin basic protein (MBP) that further activate the local T-lymphocyte population leading to ongoing tissue destruction [17;153 119].

Few studies have previously correlated gene expression in the peripheral T-lymphocyte with MS lesion pathology yet such an approach would help further elucidate the role of the T-lymphocyte in the pathogenesis of MS and may potentially help to identify a potential peripheral disease marker. Hence, we undertook to compare gene expression in MS lesions with gene expression in peripheral T-lymphocytes: initially DDD was performed to identify a cohort of genes that are dysregulated in MS brain lesions compared to normal adult brain tissue. The expression of the cohort of genes was then examined by real-time RT-PCR from mRNA extracted from human T-lymphocyte from MS patients with well-characterised MS sub-types, as defined by McDonald's criteria and compared to T-lymphocyte mRNA obtained from healthy controls [100].

4.5 Results

4.5.1 *In Silico* identifcation of MS associated genes

4.5.1.1 DDD library selection

A DDD analysis was performed using human (*Homo sapiens*) libraries only. At that time, eighteen libraries were identified. Seventeen libraries contained mRNA extracted from white and grey matter of normal adult human brain tissue, more specifically the cerebral cortex, thalamus, cerebellum and midbrain. Only a single library of mRNA extracted from MS lesions in the white matter of the cerebral cortex obtained at post mortem existed. The seventeen normal brain tissue libraries contained a total of 91,576 EST's while the single MS lesions library contained a total 11,516 EST's.

4.5.1.2 DDD output

Thirty-one EST's were identified by DDD analysis, all of which met statistical significance. Sixteen were more abundant in MS brain than normal brain tissue, while three EST's were found to be more abundant in normal brain compared to MS brain. Nine EST's demonstrated no expression in MS lesions and were only identified in normal brain (Figure 4.1). Tables 4.1 – 4.3 represent the data output from the DDD study.

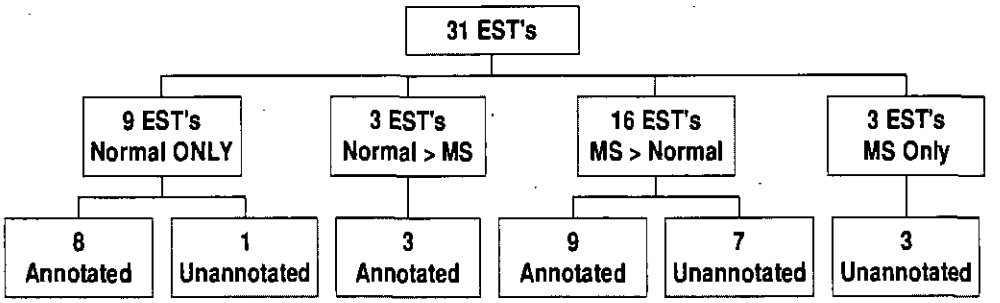


Figure 4.1. Flowchart of DDD output. mRNA extracted from whole normal brain tissue compared to mRNA extracted from MS brain lesions. The flowchart demonstrates the breakdown of expressed and annotated genes.

Twenty of the total EST's identified by DDD were annotated genes, in other words both their function and chromosomal location has previously been identified, while the remaining eleven EST's were unannotated genes (Figure 4.1). In order to characterise the eleven un-annotated genes, UniGene and Digital Extractor were used. Digital Extractor is a program that processes DDD output directly with the aim of characterising the EST sequences [258]. In a final step, all unannotated nucleotide sequences were put through BLAST to ensure correct annotation and to help further characterise the remaining unannotated sequences (http://www.ncbi.nlm.nih.gov/BLAST/). In total, seven genes were successfully characterised while the remaining four EST's were unable to be assigned to specific genes (Table 4.4).

Overall, 27/31 ESTs were successfully identified as genes and included tubulin-beta A1 (TUBA1) (Table 4.1) which has been previously shown to be expressed in MS lesions [279] and Reticulon-3 (RTN3) (Table 4.2), a poorly described

developmental gene that appears to be critical in the development of axons and glial cells [280].

Table 4.1. Genes found only in normal brain by DDD data analysis.

Gene title and symbol	Unigene Number	Accession Number
Apolipoprotein E (APOE)	Hs.169401	NM_000041
Synaptosomal-associated protein 25KD (SNAP25)	Hs.84389	NM_003081 NM_13081
Growth hormone 1 (GH1)	Hs.115352	NM_000515 NM_022559 NM_022560 NM_022561 NM_022562
Prolactin (PRL)	Hs.1905	NM_000948
Tubulin, alpha, ubiquitous (K-ALPHA-1)	Hs.334842	NM_006082
Tubulin, alpha 3 (TUBA3)	Hs.272897	NM_006009
Glycoprotein M6A (GPM6A)	Hs.75819	NM_201592
Ribosomal protein L3 (RPL3)	Hs.119598	NM_000967
Chromosome 3 open reading frame 4 (C3orf4)	Hs.107393	NM_019895

Table 4.2. Genes more abundant in normal brain versus MS brain by DDD.

Gene title and symbol	Unigene Number	Accession Number
Prion protein (p27-30) (Crutzfeld-Jakob, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia) (PRNP)	Hs.74621	NM_000311
Transmembrane 4 superfamily member 2 (TM4SF2)	Hs.82749	NM_004615
Reticulon 3 (RTN3)	Hs.252831	NM_006054 NM_201428 NM_201429 NM_201430

Table 4.3. Genes found to be more abundant in MS brain compared to control, normal brain tissue from DDD analysis.

Gene title and symbol	Unigene Number	Accession Number
Zinc-fingers and homeoboxes 1 (ZHX1)	Hs.12940	NM_007222
Annexin A1 (ANXA1)	Hs.78225	NM_000700
Nemo-like kinase (NLK)	Hs.3532	NM_016231
Myosin VI (MYO6)	Hs.22564	NM_004999
Haemoglobulin, gamma A (HBG1)	Hs.266959	NM_000559
Hepaticleukaemia factor (HLF)	Hs.250692	NM_002126
Protein phosphatase4 regulatory subunit 1 (PPP4R1)	Hs.3382	NM_005134
Progesterone receptor membrane component 2(PGRMC2)	Hs.9071	NM_006320
Tripartite motif-containing 9 (TRIM9)	Hs.75090	NM_015163 NM_052978
Homo sapien cDNA FLJ 20167 fis, clone COL09512	Hs.374538	Unknown
Homo sapien cDNA DKFZp566A1046	Hs.168950	NM_018972
KIAA1492 protein (KIAA1492)	Hs.91625	NM_001004360 NM_020868
KIAA0033 protein (KIAA0033)	Hs.174905	XM_495866
KIAA0390 protein (KIAA0390)	Hs.108884	NM_014717
Hypothetical protein FLJ10726 (FLJ10726)	Hs.268561	NM_018195
ESTs, weakly similar to S43604R07E5.1 protein, (*C.elegans*)	Hs.93828	Unknown

Table 4.4. EST's from DDD analysis annotated using UniGene and Digital Extractor

Unigene Number	Gene title and symbol
Hs.108884	Zinc finger protein 536 (ZNF536)
Hs.168950	Ganglioside-induced differentiation-associated protein 1 (GDAP1)
Hs.174905	Transmembrane protein 41B (TMEM41b)
Hs.268561	Weakly similar to cylicin I (CYLC1)
Hs.29002	Human cadherin 13 precursor (CADD)
Hs.374538	Transmembrane protein 167 (TMEM167)
Hs.91625	Dipeptidyl-peptidase 10 (DPP10)

4.5.2 Correlation of DDD output with *ex vivo* T-lymphocytes

Of the twenty-seven characterised genes identified through DDD, four were selected for further study. These genes were chosen because they were well described genes both in terms of their nucleotide sequence, function and that all four genes have been previously shown to be implicated in T-lymphocyte activation [281-284]. Two of the genes were more abundant in MS tissue, namely, Annexin-A1 (ANXA1) and Zinc-fingers-and-homeoboxes-1 (ZHX1). ANXA1 is a glucocorticoid-regulated protein that is a potent inhibitor of leukocyte trafficking in acute and chronic inflammation [285]. ZHX1 is a transcription factor that is widely expressed in human tissue and has been previously implicated in nephrotic syndrome [286].

The other two genes selected were, Apolipoprotein-E (APOE) and Prion-protein (PRNP). APOE gene codes for the lipoproteins that are the normal constituents

of blood plasma namely, chylomicrons, high density lipoprotein (HDL), and very low density lipoprotein (VLDL). With DDD analysis, APOE did not appear to be expressed in MS lesions and was only found in normal adult brain tissue. PRNP which has been implicated in neurodegenerative disorders and has a potential role in cellular stress responses of neuronal cells [287;288] was similarly found to have more marked expression in normal brain tissue compared to MS lesions.

4.5.3 Quantitative PCR *ex vivo* human T-lymphocytes

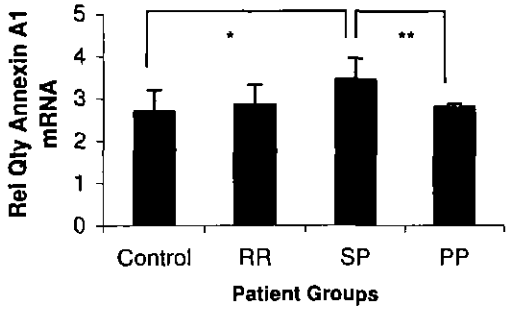
The mRNA expression of the four genes, ANXA1, ZHX1, APOE and PRNP, was analysed using quantitative PCR in human T-lymphocytes from 26 patients with different clinical phenotypes of MS and compared to healthy control T-lymphocyte mRNA (n = 3) (Figure 4.2). Of the 26 MS patients, nine had primary progressive (PP), nine had relapsing remitting (RR) and eight had secondary progressive (SP) MS. Notably, all RR MS patients were in a steady clinical state (i.e. no disease flare or acute relapse at time of blood draw) and with regard to the SP and RR phenotypes, were on disease modifying agents.

The first two genes ANXA1 and ZHX1 demonstrated increased expression in MS lesions on DDD. Real-time RT-PCR for ANXA1 demonstrated a statistically significant increase in mRNA expression in peripheral T-lymphocytes of the SP MS phenotypes compared to controls and the PP MS group (p=0.06) (p=0.03) (Figure 4.2, Panel A). While ZHX1 did demonstrate increased expression in peripheral T-lymphocytes of patients with RR MS compared to controls and the PP MS groups (p=0.01) (p =0.04) it also showed

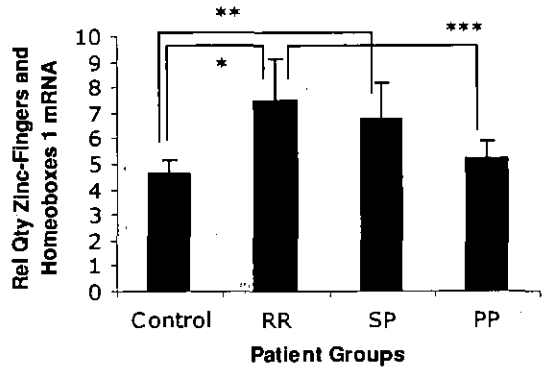
increased expression when comparing the SP MS group to controls (p=0.01) (Figure 4.2, Panel B).

For APOE and PRNP did not demonstrate altered expression in MS lesions according to DDD analysis, APOE gene expression showed significantly lower expression in the PP MS group compared to controls and the RR MS group (p= 0.02) (p=0.05) respectively (Figure 4.2, Panel C). However, PRNP did demonstrate increased expression in peripheral T-lymphocytes of patients with RR MS and SP MS compared to control with real-time RT-PCR (p=0.02) (P=0.01) (Figure 4.2, Panel D).

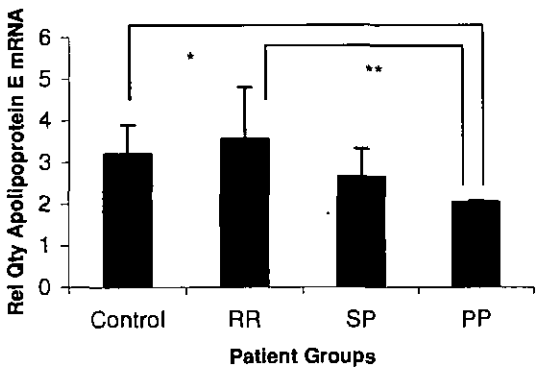
Figure 4.2 RT-PCR results of the expression of cohort of genes in T-lymphocytes



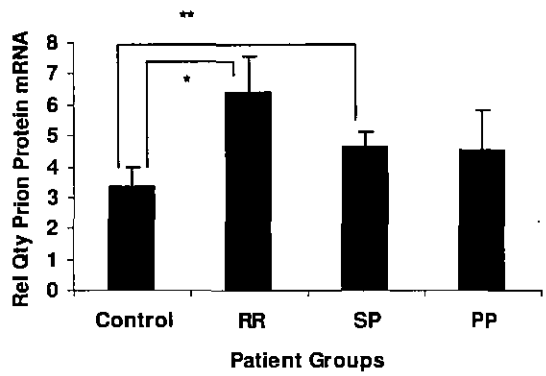
Panel A
Represents gene expression of Annexin A1 in our patient cohort (RR: relapsing- remitting, SP: secondary progressive, PP: primary progressive MS) and controls using real time RT-PCR.
**p=0.06, p=0.03*



Panel B
Represents gene expression of zinc- fingers and homeoboxes 1 in our patient cohort (RR: relapsing- remitting, SP: secondary progressive, PP: primary progressive MS) and controls using real time RT-PCR.
* p=0.01, ** P=0.01, ***p=0.04



Panel C
Represents gene expression of apolipoprotein E in our patient cohort (RR: relapsing- remitting, SP: secondary progressive, PP: primary progressive MS) and controls using real time RT-PCR.
* p=0.02, ** p=0.05



Panel D
Represents gene expression of prion protein in our patient cohort (RR: relapsing- remitting, SP: secondary progressive, PP: primary progressive MS) and controls using real time RT-PCR.
*p=0.02, ** p=001.

4.6 Discussion

MS is a polygenic inflammatory disease that may present in different clinical forms requiring different management strategies and associated with different outcomes [121]. While there have been major advances in our understanding of MS there remain large clinical deficits [289], [290], [291]. Currently, the diagnosis of MS is established through a combination of clinical, laboratory and radiological data [100]. A pathological diagnosis is rarely established in life except fortuitously in cases of acute tumoral MS where the first presentation is one of rapidly expanding mass.

The current hypothesis for the development of MS is that T-lymphocytes in genetically susceptible individuals are activated in the peripheral circulation and then migrate to CNS. In the CNS, these autoreactive T-lymphocytes release pro-inflammatory cytokines such interferon-gamma (INF-γ) and tumour necrosis factor-beta (TNF-β) that up-regulate the expression of cell-surface molecules on neighbouring lymphocytes and antigen-presenting cells (APCs) such as macrophages. The net result is further release of cytokines and chemokines leading to disruption of the blood brain barrier (BBB) thereby allowing more T- and B-lymphocytes to migrate into the CNS. The inflammatory response leads to oligodendrocyte swelling and breakdown with release of proteins such as myelin-basic protein (MBP) that can then act as an antigen to the APC and further aggravates injury. The net result of this is the formation of plaques within the white matter of the CNS [17]. Pathologically, the MS lesion comprises

of demyelinatined axons, activated macrophages, oligodendrocytes, astrocytes and infiltration of immune cells such as T-and B-lymphocytes.

In this chapter, an *in-silico* mining tool was used to identify genes that are significantly dysregulated in MS lesions. From the genes identified, where a subset that were most likely to be implicated in T-lymphocyte activation were selected and their expression was further probed: mRNA expression of these genes was quantified in human T-lymphocytes from patients with the three clinical phenotypes of MS and compared to healthy controls. By studying the correlation of gene expression between MS lesions and peripheral T-lymphocyte we aimed to further elucidate the role of the T-lymphocyte in the pathogenesis of MS and that could potentially identify disease markers that could help solve some of the deficits in the management of this most complex disease.

One of the major obstacles to defining the exact pathogenesis of MS is the limited access to CNS tissue [292;293]. DDD provides a large reservoir of previously studied gene expression data from a variety of human tissues and is therefore an invaluable starting point from which to study changes in gene expression. With DDD, mRNA extracted from a particular tissue experiment is submitted and catalogued as a library thereby facilitating the comparison of mRNA expression across such libraries. With this in mind, mRNA from normal adult brain tissues was compared to mRNA extracted from MS lesion with the aim of identifying a cohort of genes with altered expression in MS.

DDD applies strict criteria to ensure only statistically significant differences are reported when comparing gene expression across its libraries however as an *in-silico* mining tool it is important not to infer too much biological information from the results. Therefore, the expression of genes identified by a DDD search must be validated to ensure robustness [294;295].

Previous studies have shown that changes in peripheral T-lymphocyte correlate to changes in MS plaques on brain imaging [296;297]. Therefore, the results of the DDD output were used as a method to identify a small cohort of genes whose expression could be further studied in peripheral T-lymphocytes *ex-vivo*.

Four genes identified by DDD, namely Annexin A1 (ANXA1), Zinc-fingers and homeoboxes 1 (ZHX1), Apolipoprotein E (APOE) and Prion Protein (PRNP) are discussed further in the following paragraphs.

The first gene identified in MS tissue and analysed in peripheral T-lymphocytes was ANXA1. ANXA1 is a member of an anti-inflammatory superfamily of phospholipid binding proteins [298]. It is found in a variety of different cells and tissues throughout the body [299]. Interestingly, it is found within neutrophil granules where it is released on cellular adhesion to the endothelium [300;301]. Previous work has demonstrated ANXA1 to be more abundant in acute inflammation compared to chronic [302]. It has also been shown previously that ANXA1 is produced in response to steroids in some cell lines [303]. ANXA1 can bind to surface receptors on macrophages resulting in the inhibition of leukocyte diapedesis [285;304]. DDD analysis showed ANXA1 is more abundant in MS brain

tissue compared to healthy adult brain tissue a finding that is consistent with previous work where ANXA1 was found to be expressed in perivascular T-lymphocytes of active MS lesions with expression reflecting worsening disease stage [305]. Our laboratory findings showed that ANXA1 was significantly expressed in the peripheral T-lymphocytes of MS patients with SP MS compared to control and to PP MS (p=0.06, p=0.03) respectively (figure 4.2 panel A). Therefore our results did demonstrate a correlation between brain lesion gene expression of ANXA1 and peripheral T-lymphocytes of patients with SP MS phenotype perhaps pointing to a more significant inflammatory disease activity in SP MS compared to PP MS and it does support that our patients with the RR MS were in a steady clinical state (i.e. no disease flare or acute relapse at time of blood draw).

ZHX1 is the second gene selected from the DDD data analysis. No literature has previously been published on the role of ZHX1 in MS, but it has been previously shown that IL-2 specifically induces the expression of ZHX1 in mouse cytotoxic T-lymphocytes [282]. IL-2 is considered to be an important growth factor for T-lymphocytes, by inference ZHX1 plays a role in autoimmunity [306]. Our DDD analysis showed it to be present in MS brain tissue more than healthy adult brain tissue. Comparing T-lymphocyte gene expression of ZHX1 in patient's samples with different MS phenotypes to healthy controls showed a statistically significant change in RR MS and SP MS phenotypes compared to controls (p=0.01, p=0.01) respectively. However, ZHX1 expression was greater in the RR MS than in PP MS group (P=0.04) (figure 4.2 panel B). As mentioned above, ZHX1 expression is increased in activated T-lymphocytes

[282]. This is noteworthy as previous studies have suggested that peripheral T-lymphocytes play a less significant role in the pathogenesis of PP MS in which loss of oligodendrocyte and neurodegeneration seems to be the mechanism responsible for disease progression [307-309]. This is also evident as with progression there is brain atrophy as a result of axonal loss and lack of gadolinium enhancement on MRI reflecting less disruption in the BBB preventing the influx of inflammatory cells into the CNS from the peripheral circulation [289;310;311]. Thus the low expression of ZHX1 in the PP MS group in our patient mRNA compared to the RR MS and SP MS group is in keeping with the current understanding of pathogenesis of PP MS.

APOE was selected because it has been shown to play a role in the maintenance of the integrity of the BBB, which in turn is essential in the pathogenesis of MS [312]. Extensive studies have looked at the association between APOE polymorphism and MS have given no clear answer to the significance of APOE in the predisposition, onset, or disease progression and severity in patients with MS [313-316]. A study carried out by Prettila *et al* looked at the APOE levels in the cerebrospinal fluid (CSF) and serum of MS patients the study failed to identify a difference in APOE expression in both serum and CSF of MS patients [317]. With the DDD analysis APOE was identified to be abundant in normal brain tissue but absent in brain tissue taken from MS patients. Correlating this to peripheral T lymphocytes obtained from MS patients with different clinical phenotypes, our findings showed a statistically significant low expression of APOE in T-lymphocytes obtained from the PP MS group compared to controls and SP MS group (p=0.02, p=0.05) respectively (figure 4.2 panel C). It is doubtful that T-

lymphocyte APOE has a role to play in BBB integrity, however our results may reflect a generalized suppression of APOE expression in progressive MS. The trend in APOE gene expression in our patient samples was similar to that of DDD with greater expression in controls versus all MS phenotypes Interestingly, while there have been differences in APOE expression reported, as previously mentioned. Most recently, the meta analysis, which included all the major studies as with our study failed to demonstrate an association between APOE and MS [318].

The fourth and final gene selected from the DDD analysis is PRNP. PRNP has been recently reported to have superoxide dismutase- like activity and may be involved in cellular stress responses or apoptosis of neuronal cells [287;288]. It is also essential for IL-2 mRNA synthesis, therefore essential for T-lymphocyte activation [319]. The DDD data analysis showed PRNP to be more abundant in normal healthy adult brain tissue compared to MS brain tissue. The gene expression profile of PRNP in the T lymphocytes taken from patients with RR and SP MS phenotypes compared to healthy controls (p=0.02, p=0.01) respectively (figure 4.2 panel D). A trend towards increased expression in the T-lymphocytes of the MS patients may be explainable by the fact that PRNP is known to play a role in neurodegeneration (figure 4.2 panel D). The gene expression of PRNP in T-lymphocytes obtained from RR MS and SP MS patients showed statistically significant increase compared to controls. Again this is not surprising as there are neurodegenerative changes seen early in the disease [320]. Also as mentioned above PRNP is essential for IL-2 mRNA synthesis and hence essential for T-lymphocyte activation as is the case in RR

MS and SP MS. To date no study has been published in the literature examining PRNP expression in MS. However considering PRNP role in view of neurodegeneration and T-lymphocyte activation explains the results seen in our study.

4.6 Conclusion

Using DDD to explore gene expression in MS brain tissue, we identified 31 EST's that were dysregulated in cerebral MS lesions. Of those, four genes, known to play a role in T-lymphocyte activation, were chosen for further analysis. The expression of the four genes was studied in peripheral T-lymphocytes obtained from MS patients with different clinical phenotypes. Looking at the expression of these genes in peripheral T-lymphocytes we were able to demonstrate that both ZHX1 and PRNP had increased expression in the RR MS and SP MS phenotypes. The gene expression of ANXA1 was increased in the SP MS phenotype, whereas APOE gene expression was decreased in the PP MS phenotype.

DDD has been used in the past to identify candidate target genes in polygenic disorders. In this instance, DDD identified potentially interesting genes for the study of MS. However, using the more stringent gene expression analysis technique of real-time RT PCR, there was some correlation between the expression results as predicted by DDD and as measured by real-time RT PCR. This finding may be reflective of the overall complexity of the MS phenotype and also illustrate the difficulty in comparing MS tissue and lymphocyte profiles in segmented patient population.

These data demonstrate the utility of computational biology in the identification of genes associated with MS and provide a subset of T -ymphocyte associated genes whose expression is also altered in brain tissue. Further ongoing studies will more clearly elucidate the putative pathogenic activities of these genes in the initiation and progression of MS.

Chapter 5

Oligonucleotide microarray analysis of T-lymphocytes from multiple sclerosis (MS) patients and healthy controls

5.1 Aim

To examine global gene expression differences in T-lymphocytes obtained from patients with different clinical phenotypes of MS using oligonucleotide microarrays. The gene expression profiles obtained from the three different clinical MS phenotypes will be compared to those of T-lymphocytes obtained from healthy controls.

5.2 Hypothesis

We propose that differences in global gene expression profiles of T-lymphocytes may reflect key changes seen in these cells during the initiation and progression of specific MS phenotype.

5.3 Objectives

1) To examine global gene expression in MS T-lymphocyte using microarray technology. 2) To validate finding by real-time PCR (RT-PCR) and further investigate their expression in an *in vitro* model of myelin basic protein (MBP) activated T-lymphoctes.

5.4 Background and rationale

As mentioned in previous chapters, MS is a chronic inflammatory autoimmune

demyelinating disease of the central nervous system (CNS) [17]. Although investigators have shown MS to be largely a T-lymphocyte mediated autoimmune disease, its aetiology remains elusive [115;117;118;321 119].

To determine the contribution of T-lymphocytes in the pathogenetics of MS, here we employed a complementary functional genomics and computational annotation strategy (Figure 5.1). These strategies exploit the vast amount of data obtained as part of the human genome-sequencing project and the major increase in novel biomedical research technologies that have emerged over the last few decades. The completion of the Human Genome project drove the development of experimental techniques that enable the study and interrogation of the genome [322]. Of particular importance was the development of strategies for the assessment of gene expression and function, termed functional genomics. There are now a variety of techniques available to facilitate genome-wide expression profiling including differential display PCR (dd PCR)[323], suppression subtraction hybridization (SSH)[324], serial analysis of gene expression (SAGE) [325] and oligonucleotide microarray (GeneChip) technology [326]. A major limitation of all these strategies results from the large amount of data generated. Computational biology, or bioinformatics, seeks to resolve these large datasets into discrete, manageable, integrated, knowledge intense packets for the identification of those key genes and gene clusters whose differential expression drive disease responses.

Microarray technology is perhaps the most powerful of these new experimental techniques. Microarrays measure the relative abundance of messenger RNA

(mRNA) of thousands of genes in parallel [327]. When all of the mRNA of a cell is used on an array then a snapshot of the total mRNA of a cell or tissue can be obtained using this technology. Consequently, microarrays facilitate transcriptome profiling of both *in vitro* and *in vivo* models of disease in an unbiased and sensitive manner. An important component to microarray technology is that the prior knowledge of the genes is not required, thus this technology is ideally placed to identify novel genes, networks and pathways in diseased cells and tissues. The two main types of microarray systems currently in use are cDNA and cRNA oligonucleotide microarrays, the latter of which was used in the studies contained in this thesis [328].

Over the past few years, several studies have used both cDNA and oligonucleotide microarray technology to examine differential expression profiles of peripheral blood cells in multiple sclerosis (MS) patients [329;330]. Here, using Affymetrix HGU133A plus 2.0 oligonucleotide arrays, we aim to identify a cohort of genes that show dysregulated expression in T-lymphocytes of patients with three different MS clinical phenotypes, based on the Mc Donald criteria for MS and compare them to those of healthy controls [100]. Our goal is to further define the role of the T-lymphocyte in the pathogenesis of MS. This approach could provide information regarding the three different disease phenotypes and may lead to the identification of specific candidate target genes that may be implicated in disease progression.

As discussed above, gene expression profiling results in the capture of many data points pertaining to the system of interest. Computational strategies to

identify the important genes are necessary in order to rationally interrogate large data sets. Figure 5.2 shows a schematic representation of the major strategies employed to identify these key gene differences. Having annotated the disease transciptome further validation, functional assessment and disease association are necessary to identify those molecules that are potential therapeutic targets, indices of disease activity and diagnostic markers (Figure 5.3)

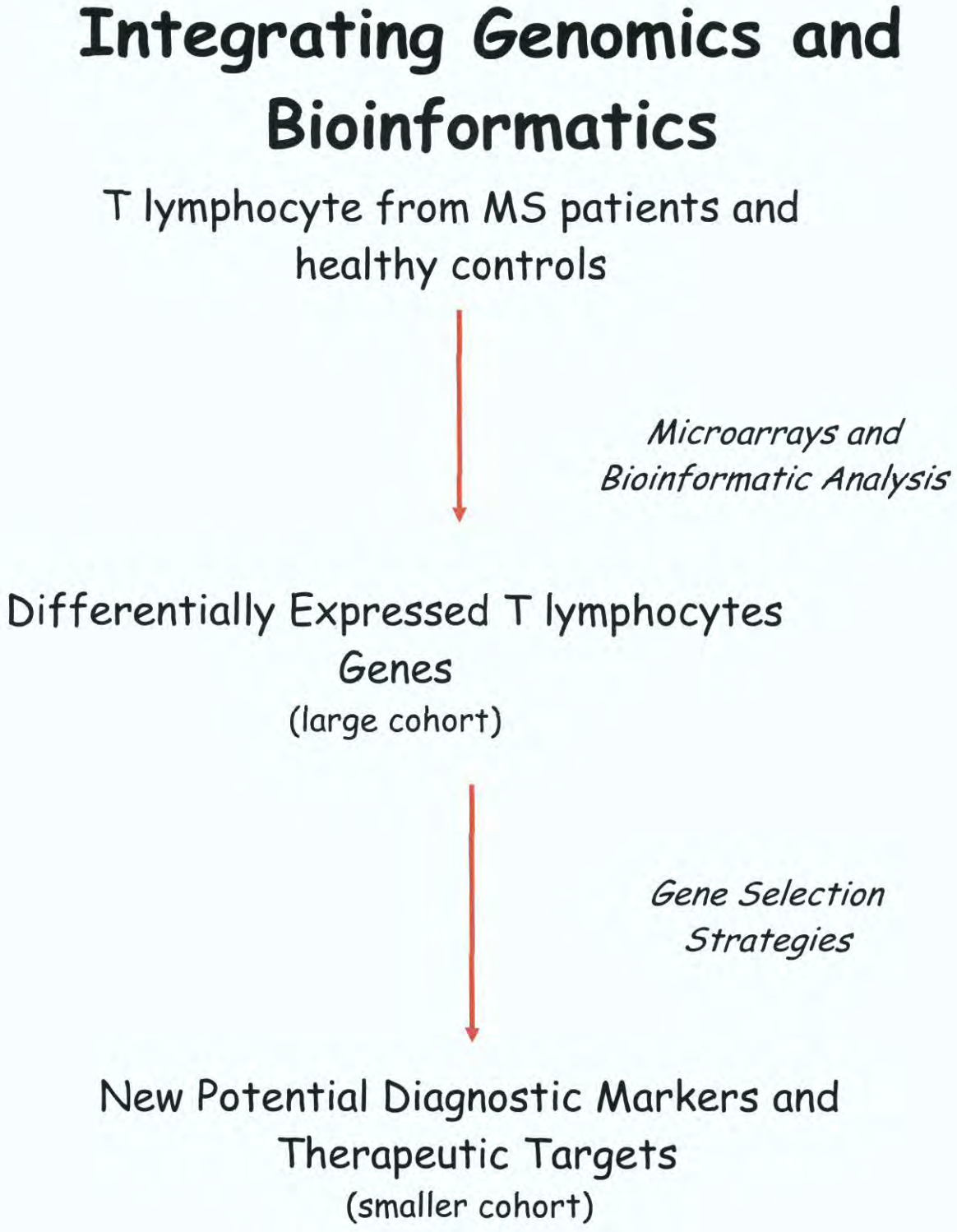


Figure 5.1. Overview of experimental approach, integrating genomics and computational approaches to the identification of MS associated genes

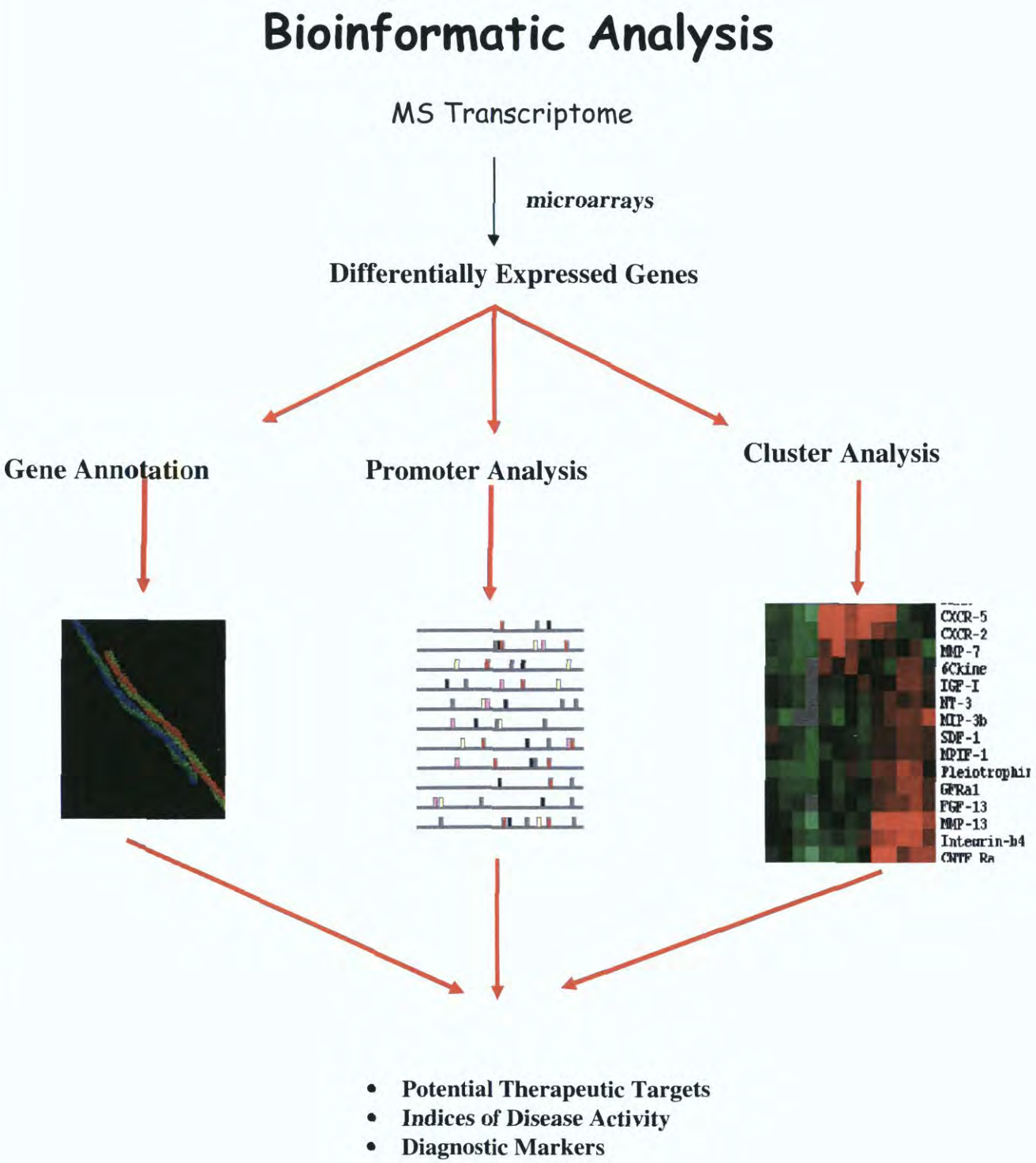


Figure 5.2. Overview of computational strategies for the annotation of oligonucleotide microarray datasets

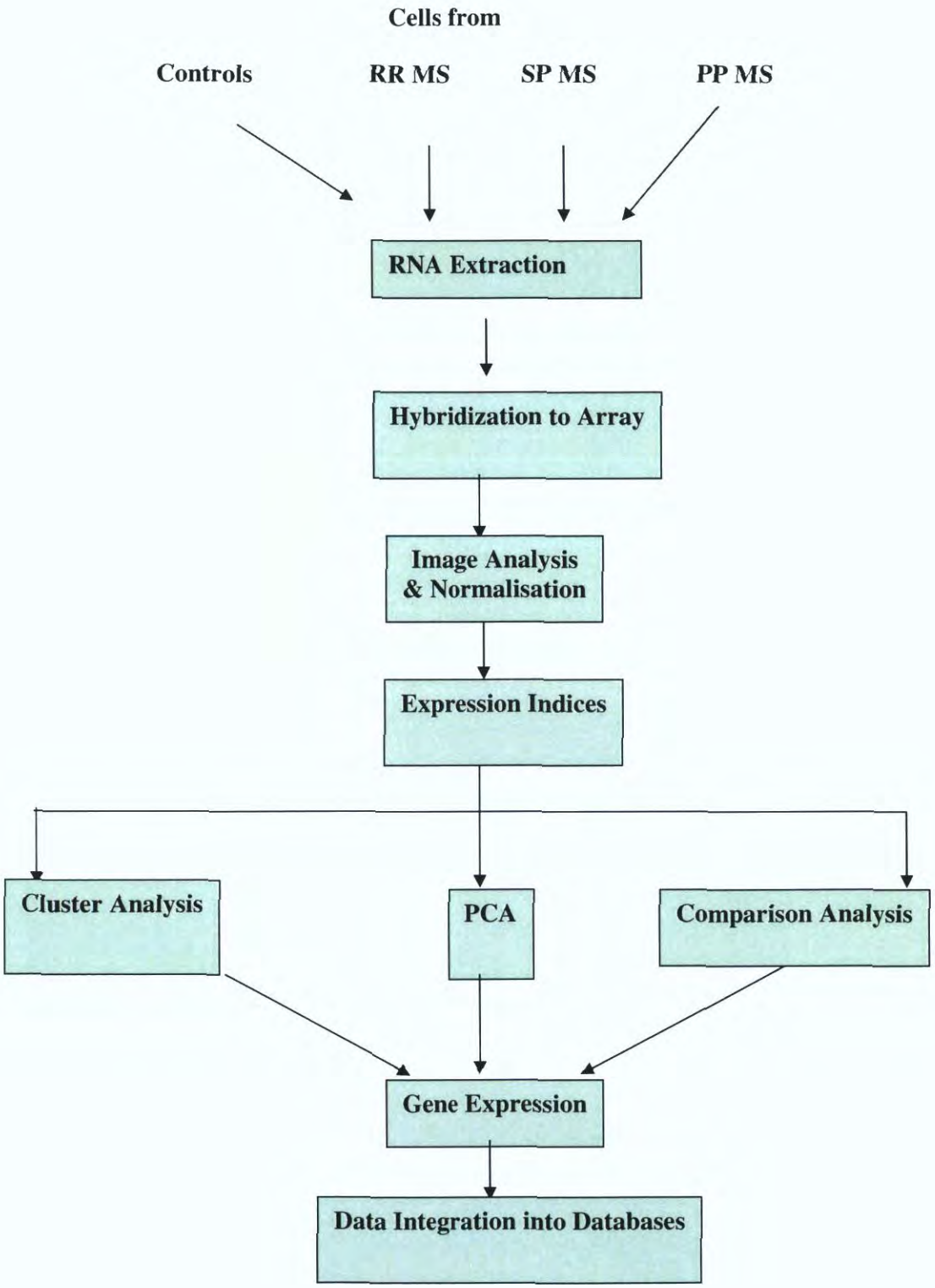


Figure 5.3. Schematic summary of microarray data analysis. RR-relapsing remitting, SP- secondary progressive, PP- primary progressive and MS- multiple sclerosis

5.5 Patient cohort

Patients with different clinical phenotypes of MS were carefully selected using the Mc Donald's criteria for MS. Healthy controls were also carefully selected for this study.

5.5.1 Patient population

A total of 11 samples were analysed using cRNA microarrays. Blood samples were taken from a) three controls, b) three relapsing- remitting and c) three secondary progressive MS and two patients with d) primary progressive MS for analysis.

5.5.2 Samples analysed

Samples from the following patients with different MS clinical phenotypes were used

in this research:

- Relapsing –remitting MS: P14, P16, P21.
- Secondary progressive MS: P15, P17 P18.
- Primary progressive MS: P19, P20.

Controls: C 14, C15, C16.

5.5.3 Patient characteristics

5.5.3.1 Relapsing- remitting MS (RR MS)

This category of MS describes patients who experience an initial exacerbation followed by complete or incomplete recovery. 85% of patients with MS follow this disease course in the ten years after their initial presentation. For our study,

we carefully selected three patients with RR MS based on the McDonald's criteria (P14, P16 and P21).

Patient P14 is a 39 year old lady who was diagnosed with MS in 2000. Her first attack with MS was in 1998, and comprised of sensory symptoms and optic neuritis. Since then she continued to have one relapse per year. Her diagnosis was confirmed with positive magnetic resonance imaging (MRI) brain findings. Her cerebrospinal fluid (CSF) analysis showed elevated protein but was negative for oligoclonal bands (OCB). In 2000, she was commenced on interferon subcutaneous injections, first with interferon beta-1a (Avonex) but subsequently on switched to interferon beta-1a (Rebif) due intolerance to the first. She was in remission when the blood sample was taken.

Patient P16 is a 39 year old lady who was diagnosed with MS in 2001. Her symptoms started in 1996 with an attack of left eye optic neuritis that fully resolved. Her second attack of MS was in 2001 with right eye optic neuritis, a third attack was in 2001 with left eye symptoms and her most recent attack in 2002 with right eye optic neuritis and right upper limb weakness. Her diagnosis was confirmed with positive brain MRI findings and also positive CSF findings for OCB. In 2001, she was commenced on interferon beta-1b (Betaseron) subcutaneous injections. She was in remission when the blood sample was taken.

Patient P21 is a 46 year old gentleman who was diagnosed with MS in 1998 with left lower limb weakness and paresthesia, he also had urinary urgency. He

had a second attack in 1999 with right lower limb paresthesia and worsening urinary symptoms. He still has residual deficit in his left lower limb since the first attack in 1998. His diagnosis was confirmed with positive MRI brain findings, positive CSF findings with OCB and positive visual evoked potentials (VER). He has been treated with interferon beta-1a (Avonex) since 2000. Of note he has 10 siblings of which three (a brother and a two sister) have MS.

5.5.3.2 Secondary progressive MS (SP MS)

50% of patients presenting with RR MS, will eventually develop gradual progression of disability that may or may not be accompanied by exacerbations. For our study, we carefully selected three patients with SP MS based on the McDonald's criteria (P15, P17 and P18).

Patient P15 is a 61 year old gentleman who was diagnosed with MS in 1998. His first symptom started in 1987 with optic neuritis. His second attack was in 1991 with right foot drop and since his symptoms progressed and eventually he became wheelchair bound with expanded disability status (EDSS) of 6.5. At the time, his diagnosis was confirmed with positive MRI brain findings; unfortunately his CSF findings are not available. In 1995 he was commenced on interferon beta-1b (Betaseron) subcutaneously which was then switched to glatiramer acetate (Copaxone) in 2002 due to progression of his symptoms. Of note he is also being treated with atorvastatin (Lipitor) for hypercholestrolaemia.

Patient P17 is a 34 year old gentleman who was diagnosed in 1999 with MS. His symptoms started with left eye optic neuritis. In 2001, he had his second attack with unsteady gait, decreased left lower limb coordination, impotence and

urinary symptoms. At the time, his diagnosis was confirmed with positive MRI brain findings and positive CSF findings for OCB. He was commenced on interferon treatment interferon beta-1a (Avonex) in 2001, swiched to interferon beta-1a (Rebif) in 2004 and then to glatiramer acetate (Copaxone) in 2005. Due to the deterioration of his symptoms, he was treated with immunosupressant (mitoxantrone) for six months in 2003, a course of intravenous immunoglobulin in 2004 and plasmaphoresis in 2005. Since, his symptoms continued to progress and eventually he became wheelchair bound with EDSS of 6.0.

Patient P18 is a 52 year old gentleman who was diagnosed with MS in 1999. His first presentation was optic neuritis. Since 2002, his neurological condition began to progress with EDSS of 3.0. At the time, his diagnosis was confirmed with a positive MRI brain and spine, positive CSF findings for OCB and also positive somatosensory evoked potentials (SSEP). At the time of diagnosis, he was commenced on interferon beta-1a (Betaseron) and continued taking it for a period of eighteen months only. He stopped taking his interferon due to the continuous deterioration of his neurological symptoms and in 2003 he was given a six months course of intravenous immunosuppressant (mitoxantrone).

5.5.3.3 Primary progressive MS

10% of patients diagnosed with MS experience a gradual progression of disability from the time of onset that is not accompanied by exacerbations. For our study, we carefully selected three patients with PP MS based on the McDonald's criteria (P19 and 20).

Patient P19 was a 62 year old lady, was diagnosed in 1995. Her diagnosis was confirmed with positive MRI brain findings and positive VER findings. Since the time of her diagnosis, her neurological symptoms continued to progress, she eventually became wheelchair bound with EDSS of 7.5. Between 2000-2001, she was treated with different courses of immunosupressants (azathioprine, methotrexate and cyclophosphamide). She passed away a year ago.

Patient P20 is a 37 year old gentleman, was diagnosed with MS in 1992. His symptoms started in 1992 with left lower limb weakness. The following year both his lower limbs became weak and since his symptoms continued to progress. Currently he is wheelchair bound with EDSS of 7.5. At the time, his diagnosis was confirmed with positive MRI brain and spine, CSF and also VER findings. At the start of his diagnosis he was treated with interferon beta 1*b* (Betaseron) for five years. Also in 1997, he was treated with immunosupressant (azothioprine) for six weeks and in 2002 he was given a six weeks course of intravenous immunosupressant (mitoxantrone). His condition is continually deteriorating.

Blood was also taken from a third patient with PP MS, but due to the poor quality of the RNA extract, the sample was ommitted from further analyses.

5.5.3.4 Controls

Three healthy individuals were selected as controls subjects for our study.

5.6 Gene expression analysis in MS

In our study, we used Tri-Reagent (trizol) to extract RNA from T-lymphocytes obtained from MS patients with different clinical phenotypes and controls, as previously described in chapter 3. Total RNA was used for subsequent analysis using oligonucleotide microarrays and for real time RT-PCR. The qualitative assessment of RNA was carried out using denaturing gel electrophoresis prior to the use of samples in these analyses. RNA, with 18S and 28S ribosomal RNA bands at approximate ratio of 2:1 was used (figure 3.2 chapter 3). Quantification of the samples was performed using an Agilent 2100 bioanalyzer RNA 6000 nanochip assay (Conway Institute Biomolecular and Biomedical Research, University College Dublin). A representative result is shown in figure 3.3 (chapter 3). The dominant spikes in the picture represent the amount of 28S and 18S rRNA in the sample. The strategies permitted the selection of high quality RNA for subsequent analysis.

5.7 Summary of gene expression changes

Data generated from samples taken from patients with RR MS were compared to data taken from healthy controls, the results showed 178 genes were significantly upregulated and 506 genes were significantly downregulated. Data generated from samples taken from patients with SP MS were compared to data taken from healthy controls, the results showed 105 genes were significantly upregulated and 273 genes were significantly down-regulated. Data generated from samples taken from patients with PP MS were compared to healthy controls, the results showed 246 genes were significantly upregulated and 314 were significantly downregulated. The summary of the data generated from the

microarrays analysis of the different clinical phenotypes of MS vs controls are as follows:

	Number of genes that are significantly altered	
	Upregulated	Downregulated
RR vs controls	178 genes	506 genes
SP vs controls	105 genes	273 genes
PP vs controls	246 genes	314 genes

The following tables (1,2,3,4,5 and 6) are the tables of the top twenty upregulated and down-regulated genes when comparing RR, SP and PP samples *vs controls*. Functional annotation was completed using an internet tool: DAVID (Database for Annotation, Visualisation and Integrated Discovery) freely accessible from the National Institute of Allergy and Infectious Disease (http://david.abcc.ncifcrf.gov/). The final lists of significantly upregulated and downregulated genes from each data was submitted into DAVID website. GOTERM-BP (biological function) and GOTERM-MF (molecular function) were selected for the functional annotation of the genes.

On the following pages, results of microarray analyses of each disease phenotype versus control are presented.

5.7.2 Relapsing- remitting MS (RR MS) transcriptome

5.7.1a Gene expression changes

Microarray data from patients with RR MS blood samples were compared to data taken from blood samples taken from control, as described above. This

analysis identified 178 genes whose expression was significantly upregulated and 506 genes whose expression was significantly downregulated in comparison with control samples.

Tables 5.1 and 5.2 represent the top twenty upregulated and downregulated genes respectively when comparing the data from patient samples with RR MS vs controls. The following is a summary of function of some of those genes.

Probeset	Gene	Symbol	SLR	p-value
222330_at	ERO1-like (S.cerevisiae)	ERO1L	2.08	0.03
211302_s_at	Phosphodiesterase 4B, sAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	PDE4B	1.93	0.01
210379_s_at	tousled-like kinase 1	TLK1	1.61	0.01
215483_at	A kinase (PRKA) ankor protein (yotiao) 9	PRKAR2A	1.58	0.05
233121_at	transmembrane protein 2	TMEM2	1.55	0.05
1557166_at	programmed cell death 4 (neoplastic transformation inhibitor)	PDCD4	1.54	0.03
214615_at	purinogenic receptor P2Y, G-protein coupled, 10	P2RY10	1.52	0.04
1555632_at	Hepatocyte growth factor-like protein	HGFL	1.50	0.00
202973_x_at	family with sequence similarity 13, member A1	FAM13A1	1.44	0.05
1555938_x_at	vimentin	VIM	1.43	0.04
205214_at	serine/threonine kinase 17b (apoptosis-inducing)	STK17B	1.37	0.00
1569053_at	adaptor-related protein complex 3, mu 2 subunit	AP3M2	1.32	0.04
244035_at	myeloid/lymphoid or mixed-lineage leukaemia (trithorax homolog, Drosophila)	MLL	1.29	0.04
1559203_s_at	v-Ha-ras Harvey rat sarcoma viral oncogene homlog	HRAS	1.28	0.00
206108_s_at	splicing factor, arginine/serine-rich 6	SFRS6	1.27	0.03
206341_at	interleukin 2 receptor, alpha	IL2RA	1.18	0.02
1559691_at	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	NDUFS1	1.15	0.02
239861_at	capping protein (actin filament) muscle Z-line, alpha 2	CAPZA3	1.13	0.05
234149_at	inositol 1,4,5-trisphosphate 3-kinase B		1.12	0.01
203725_at	Growth arrest and DNA-damage-inducible, alpha	GADD45A	1.07	0.04

Table 5.1. The top twenty upregulated genes from the microarray data when comparing samples from patients with RR MS vs controls.
(SLR-signal log ratio)

Probeset	Gene	Symbol	SLR	p-value
216191_s_at	Tcell receptor delta locus		-3.33	0.01
224516_s_at	CXXC finger 5	CXXC finger 5	-1.95	0.00
209662_at	centrin, EF-hand protein, 3 (CDC31 homolog, yeast)	CETN3	-1.81	0.01
206978_at	chemokine (C-C motif)receptor 2	CCR2	-1.79	0.02
217078_s_at	leukocyte membrane antigen		-1.77	0.01
212592_at	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha		-1.77	0.05
207840_at	CD160 antigen	CD160	-1.73	0.03
228245_s_at	ovostatin	OVOS	-1.68	0.03
240573_at	chromosome 9 open reading frame 64		-1.66	0.44
204105_s_at	neuronal cell adhesion molecule	NRCAM	-1.65	0.03
203470_s_at	pleckstrin	PLEK	-1.54	0.04
232182_at	3'(2'), 5'-bisphosphonate nucleotidase 1		-1.49	0.3
230836_at	zinc finger protein 148 (pHZ-52)	ZNF148	-1.41	0.04
1555691_a_at	killer cell lectin-like receptor subfamily C, member 4	KLRC4	-1.38	0.00
212405_s_at	CGI-01 protein	CGI-01	-1.37	0.03
210387_at	histone 1, H2bg	HIST1H2BG	-1.37	0.02
201939_at	polo-like kinase 2 (Drosophila)	PLK2	-1.35	0.03
204125_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly family	NDUFA1	-1.33	0.01
210288_at	killer cell lectin-like receptor subfamily G, member 1	KLRG1	-1.33	0.04
209585_s_at	multiple inositol polyphosphate histidine phosphate, 1	MINPP1	-1.31	0.05

Table 5.2. The top twenty downregulated genes from the microarray data when comparing samples from patients with RR MS vs controls.
(SLR-signal log ratio)

5.7.1b Functional annotation

Having identified a cohort of genes whose expression was significantly altered in RR MS when compared to control samples, we sought to determine the functional relevance of these array identified MS associated genes.

We categorized the significantly perturbed genes according to their biological function. This approach permits rapid annotation of large datasets for the identification of functional patterns of dysregulation. All significantly perturbed genes were used as input in classification searches.

Figure 5.4 shows the number of genes involved in the biological processes using GOTERM analysis. A significant number of genes were found to be downregulated for biological pathways such as cell growth and maintenance and response to stress in the RR MS patient samples compared to controls. Where as less number of genes were upregulated for the same processes.

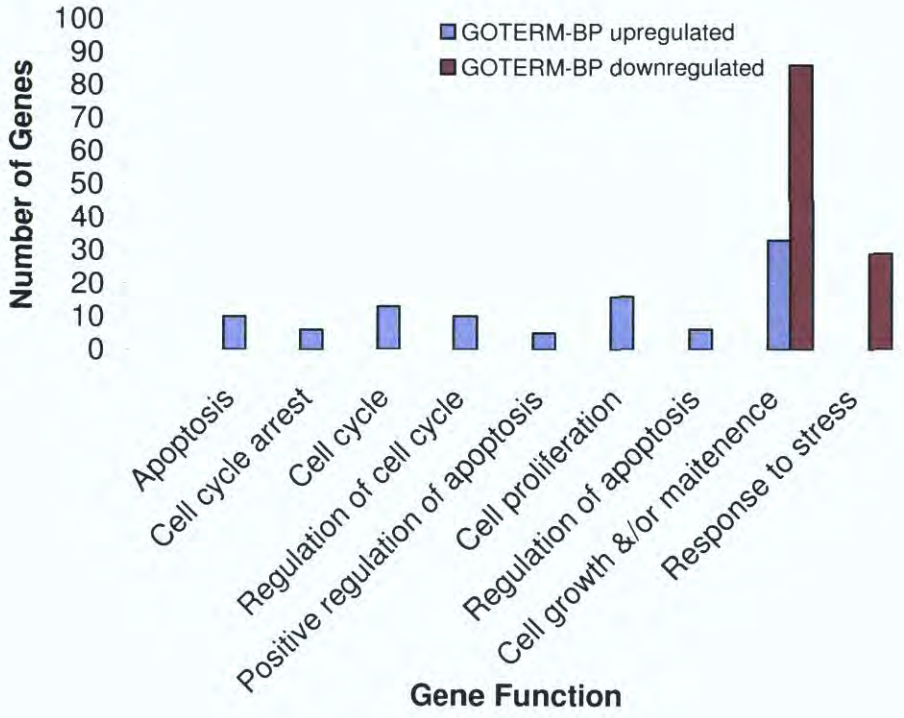


Figure 5.4. Functional Annotation of RR MS Transcriptome
Genes that are upregulated and downregulated in RR MS compared to controls-using DAVID-GOTERM biological pathway data analysis of the microarray samples

5.7.2 Secondary Progressive MS (SP MS) transcriptome

5.7.2a Gene expression changes

Microarray data from patients with SP MS blood samples were compared to data taken from blood samples taken from control, as described above. This analysis identified 105 genes whose expression was significantly upregulated and 273 genes whose expression was significantly downregulated in comparison with control samples.

Tables 5.3 and 5.4 represent the top twenty upregulated and downregulated genes respectively when comparing the data from patient samples with SP MS vs controls. The following is a summary of function of some of those genes.

Probeset	Gene	Symbol	SLR	P-value
202917_s	S100 calcium binding protein A8 (calgranulin A)	S100A8	4.346809	0.008376
212998_x	major histocompatibility complex, class II, DQ beta1	HLA-DQB1	1.666979	0.039596
211506_s	interleukin 8	IL8	1.386639	0.02025
214657_s	Trophoblast-derived noncoding RNA	TncRNA	1.252991	0.026602
1562255_at	Synaptotagmin-like 3	SYTL3	1.230868	0.037647
213975_s	Lysozyme	LYZ	1.22449	0.026657
226736_at	churchill domain containing 1	CHURC1	1.213929	0.044106
211302_s	Phosphodiesterase 4B, cAMP-specific	PDE4B	1.189317	0.039297
209619_at	CD74 antigen	CD74	1.172846	0.038577
205033_s	defensin, alpha 3, neutrophil specific	DEFA3	1.144211	0.029379
1569053_at	adaptor-related protein complex 3, mu 2 subunit	AP3M2	1.120201	0.024374
239163_at	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	UBE2B	1.111689	0.014271
224917_at	likely ortholog of rat vacuole membrane protein 1	VMP1	1.100103	0.000755
203231_s	ataxin 1	ATAXN1	1.089599	0.015636
234149_at	Thyroid hormone receptor associated protein 3	THRAP3	1.080706	0.010231
204015_s	dual specificity phosphatase 4	DUSP4	1.031481	0.03996
1565804_at	Homeobox C14	LOC36003	1.020327	0.009219
21792_s	PR domain containing 1, with ZNF domain	PRDM1	1.003082	0.005949
226818_at	macrophage expressed gene 1	MPEG1	0.984604	0.00801
232213_at	Pellino homolog 1 (Drosophila)	HLA-DQA1	0.982172	0.031599

Table 5.3. The top twenty upregulated genes from the microarray data when comparing samples from patients with SP MS vs controls.
(SLR-signal log ratio)

Probeset	Gene	Symbol	SLR	P-value
1552625_at	tRNA nucleotide transferase,	TRNT1	-1.89545	0.029529
1552733_at	kelch domain containing 1	KLHDC1	-1.656061	0.034191
1552875_at	CD200 receptor 1	CD200R1	-1.463318	0.018454
1554609_at	similar to Cytochrome c, somatic	MGC12965	-1.450446	0.002053
1555247_at	Rap guanine nucleotide exchange factor (GEF) 6	RAPGEF6	-1.384126	0.012196
1555275_at	kelch-like 6	KLHL6	-1.309428	0.022318
1556054_at	TBC1 domain family, member 8 (	TBC1D8	-1.212167	0.004542
1557706_at	zinc fingers and homeoboxes 2	ZHX2	-1.165249	0.048884
1563080_at	chromosome 13 ORF 24	C13orf24	-1.106418	0.003865
200768_s	methionine adenosyltransferase II, ALPHA	MAT2A	-1.06682	0.022229
200875_s	nucleolar protein 5A (56Kda with KKE/D repeat)	NOL5A	-1.064866	0.038353
200877_at	chapronin containing TCP1, subunit 4 (delta)	CCT4	-1.063902	0.012943
201695_s	nucleoside phosphorylase	NP	-1.058172	0.013187
201849_at	BCL2/ adenovirus E1B 19kDa interacting protein 3	BNIP3	-1.057778	0.003494
201889_at	family with sequence similarity 3, member C	FAM3C	-1.052112	0.003412
201946_s	chaprone containing TCP1, subunit 2 (beta)	CCT2	-1.0369	0.026432
20256_at	Karyopherin alpha 1 (importin alpha 5)	KPNA1	-1.029212	0.048511
202060_at	SH2 domain binding protein 1 (tetratricopeptide repeat containing)	SH2BP1	-1.025745	0.021277
202502_at	Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	ACADM	-1.025428	0.006454
202630_at	Amyloid beta precursor protein (cytochrome tail) binding protein 2	APPBP2	-1.023331	0.017546

Table IV. The top twenty downregulated genes from the microarray data when comparing samples from patients with SP MS vs controls.
(SLR-signal log ratio)

5.7.2b Functional annotation

Having identified a cohort of genes whose expression was significantly altered in SP MS when compared to control samples, we sought to determine the functional relevance of these array identified MS associated genes.

We categorized the significantly perturbed genes according to their biological function. This approach permits rapid annotation of large datasets for the identification of functional patterns of dysregulation. All significantly perturbed genes were used as input in classification searches.

Figure 5.5 shows the number of genes involved in the biological processes using GOTERM analysis. Interestingly a significant number of genes were found to be upregulated for biological pathways such as immune response, cell growth and maintenance and cell response to stimuli in the SP MS patient samples compared to controls. Whereas a larger number of genes were downregulated for biological pathways such as cell cycle.

Figure 5.6 shows the number of genes in different categories of molecular functions using GOTERM analysis. Again a significant number of genes were upregulated for the SP MS patient samples in comparison to controls for functions such as interleukin receptor activity. No genes were downregulated for the same function in comparison to controls.

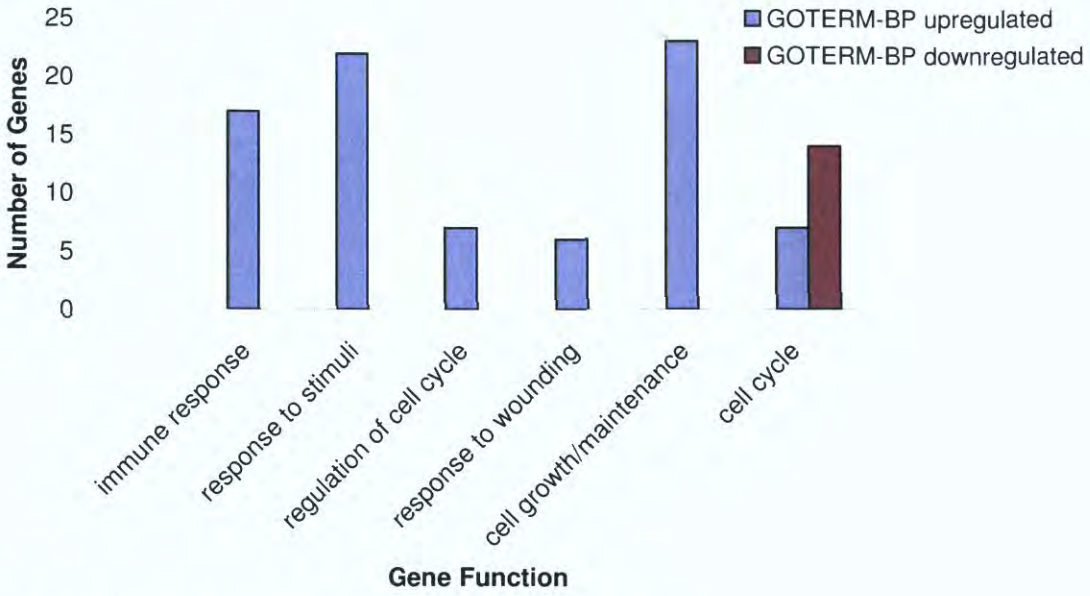


Figure 5.5. Functional Annotation of SP MS Transcriptome
Genes that are upregulated and downregulated in SP MS compared to controls- using DAVID-GOTERM biological pathway data analysis of the microarray samples.

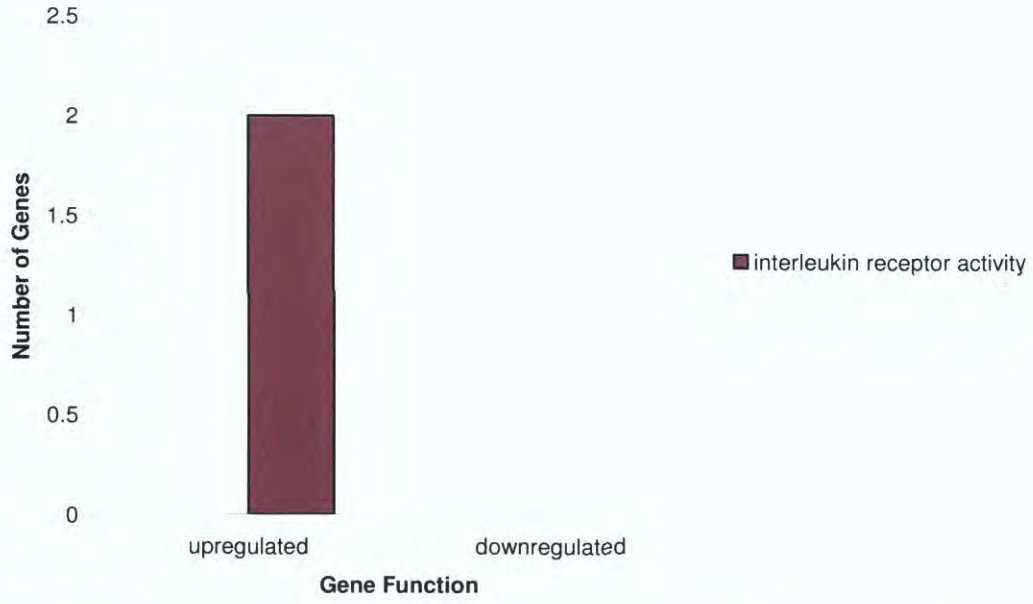


Figure 5.6. Functional Annotation of SP MS Transcriptome
Genes that are upregulated and downregulated in secondary progressive MS compared to controls using DAVID-GOTERM molecular function data analysis of the microarray samples.

5.7.3 Primary progressive MS (PP MS) transcriptome

5.7.3a Gene expression changes

Microarray data from patients with PP MS blood samples were compared to data taken from blood samples taken from control, as described above. This analysis identified 246 genes whose expression was significantly upregulated and 314 genes whose expression was significantly downregulated in comparison with control samples.

Tables 5.5 and 5.6 represent the top twenty upregulated and downregulated genes respectively when comparing the data from patient samples with PP MS vs controls.

Probeset	Gene	Symbol	SLR	P-value
202917_s	S100 calcium binding protein A8 (calgranulin A)	S100A8	5.461206	0.010014
201169_s	basic helix-loop-helix containing, class B2	BHLHB2	2.336074	0.002423
213975_s	lysozyme	LYZ///LILRB1	2.294054	0.000935
215671_at	Phosphodiesterase 4B, cAMP-specific	PDE4B	2.238243	0.002153
1555745_at	lysozyme (renal amyloidosis)	LYZ	2.061179	0.000815
1562255_at	Synaptotagmin-like 3	SYTL3	2.043504	0.016759
217739_s	pre-B-cell colony enhancing	PBEF1	2.017483	0.008126
225262_at	FOS-like antigen 2	FOSL2	1.983832	0.029056
207735_at	ring finger protein 125	RNF125	1.932926	0.010948
203535_at	S100 calcium binding protein A9 (calgranulin B)	S100A9	1.867766	0.040512
1569053_at	adaptor-related protein complex 3, mu 2 subunit	AP3M2	1.854946	0.038456
217047_s	family with sequence similarity 13, member A1	FAM	1.831209	0.042047
1555938_x	vimentin	VIM	1.818217	0.000735
1570507_at	splicing factor, arginine/serine- rich 2, interacting protein	SFRS2IP	1.817841	0.041007
213056_at	FERM domain containing 4	FRMD4B	1.815631	0.030106
223674_s	CDC42 small effector 1	CDC42SE1	1.805504	0.015715
1555749_at	splicing factor	SF1	1.804155	0.032662
232213_at	Pellino homolog 1	PELI1	1.752921	0.000439
203869_at	ubiquitin specific proteases	USP46	1.74505	0.030825

Table 5.5. The top twenty upregulated genes from the microarray data when comparing samples from patients with PP MS vs controls.
(SLR-signal log ratio)

Probeset	Gene	Symbol	SLR	P-value
231798_at	noggin	NOG	-2.501194	0.002206
232315_at	Zinc finger-like	LOC40071	-1.983573	0.015949
209662_at	centrin, EF-hand protein, 3 (CDC31 homlog, yeast)	CETN3	-1.87926	0.018788
224797_at	arrestin domain containing 3	ARRDC3	-1.76743	0.004082
204105_s	neuronal cell adhesion molecule	NRCAM	-1.708216	0.037006
209841_s	leucine rich repeat neuronal 3	LRRN3	-1.69935	0.016276
235306_at	GTPase, IMAP family member 8	GIMAP8	-1.482064	0.041748
232164_s	epiplakin 1	EPPK1	-1.451511	0.045848
215743_at	N-myristoyltransferase 2	NMT2	-1.43425	0.021733
203403_at	armadillo repeat containing, X-	ARMCX2	-1.409637	0.000853
227486_at	5'-nucleotidase, ecto (CD73)	NT5E	-1.363002	0.004983
222500_at	peptidylpropyl isomerase (cyclophilin)-like 1	PPIL1	-1.336743	0.037509
212407_at	CGI-01 protein	CGI-01	-1.326523	0.46861
213652_at	proprotein convertase subtilisin/ kexin type 5	PCSK5	-1.276473	0.003699
226683_at	sorting nexin associated golgi protein 1	SNAG1	-1.259549	0.02758
226996_at	lysocardiolipin acyltransferase	LYCAT	-1239051	0.009792
218129_s	nuclear transcription factor Y, beta	NFYB	-1.195819	0.028267
219777_at	GTPase, IMAP family member 6	GIMAP6	-1.195611	0.047574
226604_at	SMILE protein	SMILE	-1.181519	0.04139
204125_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	NDUFAF1	-1.169497	0.036375

Table 5.6. The top twenty downregulated genes from the microarray data when comparing samples from patients with PP MS vs controls.
(SLR-signal log ratio)

5.7.3b Functional annotation

Figure 5.7 shows the number of genes involved in the biological processes using GOTERM analysis. A significant number of genes were found to be upregulated for biological processes such as signal transduction, transcription and apoptosis in the PP MS patient samples compared to controls. Where as much less number of genes were downregulated for processes such as apoptosis, protein biosynthesis and apoptosis.

Figure 5.8 shows the number of genes involved in molecular function using GOTERM analysis. Again a significant number of genes were upregulated for the PP MS patient samples in comparison to controls for processes such as protein and nucleic acid binding where as fewer genes were downregulated for processes such as DNA binding.

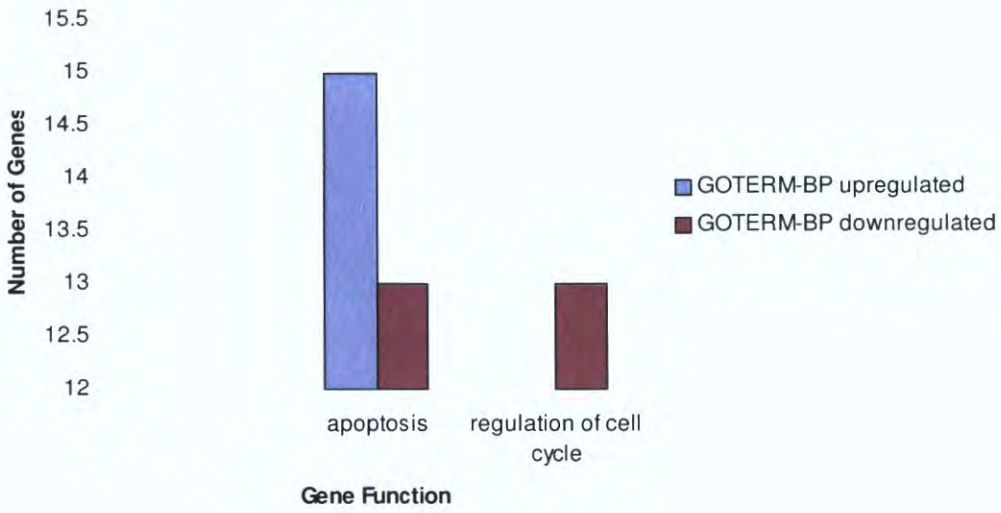


Figure 5.7. Functional Annotation of PP MS Transcriptome
Genes that are upregulated and downregulated in PP MS compared to controls- using DAVID-GOTERM biological pathway data analysis of the microarray samples.

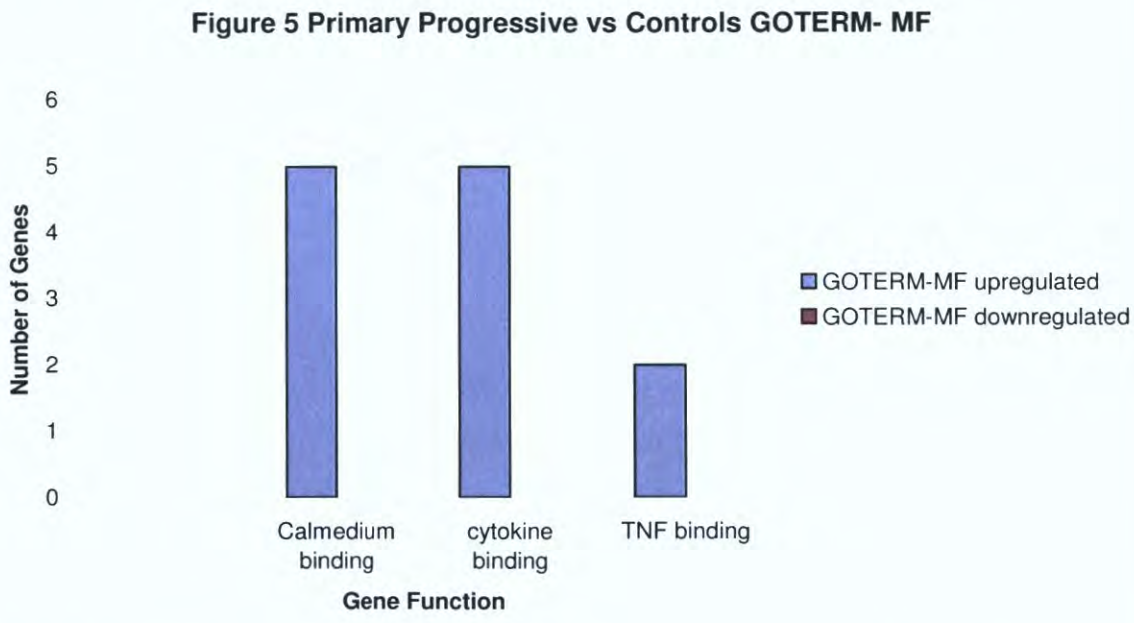


Figure 5.8. Functional Annotation of PP MS Transcriptome
Genes that are upregulated and downregulated in PP MS compared to controls- using DAVID-GOTERM molecular function data analysis of the microarray samples.

5.8 Comparing top twenty genes between the different groups

A comparative examination of the tables (table 5.7 and 5.8) listing the top twenty upregulated and downregulated genes in each disease (RR, SP, and PP MS) was carried out.

5.8.1 Genes found within top twenty of both SP and PP groups.

A number of genes were found to be similarly upregulated versus control in SP and PP groups. S100A8 was the highest upregulated gene in both SP and PP groups (SLR 4.34 and 5.46 respectively). SYTL3 was increased by 1.23 SLR in SP (5^{th} of 20) and 2.04 in PP (6^{th} of 20). Lysozyme was found to be upregulated by 1.22 SLR in SP (6^{th} of 20) and by 2.06 in PP samples (3^{rd} of 20). Expression of the above genes was not significantly different between RR and control groups. (table 5.7A)

5.8.2 Genes found within top twenty of all groups (RR, SP and PP)

Two genes were found to co-exist as members of the top twenty upregulated genes across all disease categories. These gene were PDE4B which showed a 1.93 SLR change in RR (2^{nd} of 20), a 1.18 SLR change in SP (8^{th} of 20) and a 2.23 SLR change in PP (4^{th} of 20). AP3M2 was found to be increased by 1.32 SLR in RR (12^{th} of 20), 1.12 SLR in SP (9^{th} of 20) and 1.85 SLR in PP (11^{th} of 20). (table 5.7B)

5.8.3 Genes found within top twenty downregulated of RR and PP

Three genes were found to be similarly downregulated versus control in RR and PP groups. NRCAM was downregulated by – 1.65 SLR in RR (10^{th} of 20) and –

1.70 SLR in PP (5th of 20). Additionally NRCAM (although not in the top twenty) was also significantly downregulated in SP group SLR - 0.644 (241st of a total of 275 downregulated genes in SP). CGI-01 was downregulated by – 1.37 SLR in RR (15th of 20) and – 1.32 in PP (13th of 20). NDUFAF1 was found to downregulated by – 1.33 SLR in RR (18th of 20) and by – 1.16 in PP samples (20th of 20). Expression of the above genes was not significantly different between SP and control groups. (table 5.8)

The following tables (table 5.7 and 5.8) represent the list of those genes.
A)

Gene	Symbol	MS Category	SLR	Behaviour in RR	Potential as modulator of T-cell function in MS
S100 calcium binding protein A8 (calgranulin A)	S100A8	SP PP	4.346809 5.461206	No sig. diff. vs C	*IFN-γ regulated gene *Activates lymphocytes
Synaptotagmin-like 3	SYTL3	SP PP	1.230868 2.043504	No sig. diff. vs C	*Promotes chemotactic T lymphocyte migration.
Lysozyme	LYZ	SP PP	1.22449 2.061179	No sig. diff. vs C	No evidence of role to date

B)

Gene	Symbol	Potential as modulator of T-cell function in MS
Phosphodiesterase 4B, cAMP-specific	PDE4B	Induced in T-cell activation Role in T-cell proliferation
Adaptor-related protein complex 3, mu 2 subunit	AP3M2	*Clathrin vesicle formation* Internalization/recycling of receptor complexes

Table 5.7 A) Genes found to be among the top twenty upregulated versus C in SP and PP MS categories. B) Genes found to be among the top twenty upregulated versus C in all MS categories.

Gene	Symbol	MS Category	SLR	Behaviour *in SP*	Potential as *modulator* of T-cell function in MS
Neuronal cell adhesion *molecule*	NRCAM	RR PP	- 1.65 - 1.70	No sig. diff vs C	No evidence in literature
CGI-01 protein	CGI-01	RR PP	- 1.37 -1.32	No sig. diff vs C	Hypothetical gene No ascribed function
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly family member	NDUFA1	RR PP	- 1.33 - 1.16	No sig. diff vs C	No specific correlation with T-cells Essential gene in oxidative phosphorlyation

Table 5.8. Genes found to be among the top twenty downregulated versus C in RR and PP MS categories

5.9 Genes Selected for further analysis

For further validation studies we selected five genes that were found to be upregulated in our secondary progressive MS patient's samples.

5.9.1 SAM domain and HD domain 1

Also called: Dendritic cell- derived IFNγ-induced protein; DCIP. Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) able to induce a primary T-lymphocyte response in vivo. With maturation, DCs shift from antigen-uptake and -processing functions to an antigen-presentation phenotype. DCIP is found in all tissues except brain and thymus. It is located on chromosome 20q11.22 [331].

Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of SAMHD1 in the SP MS patient group using GOTERM analysis.

To confirm the microarray identified alterations in SAMHD1 in MS, real time RT-PCR analysis was completed. As can be seen in Figure 5.9 (A), SAMHD1 mRNA levels are significantly increased in both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured SAMHD1 transcript.

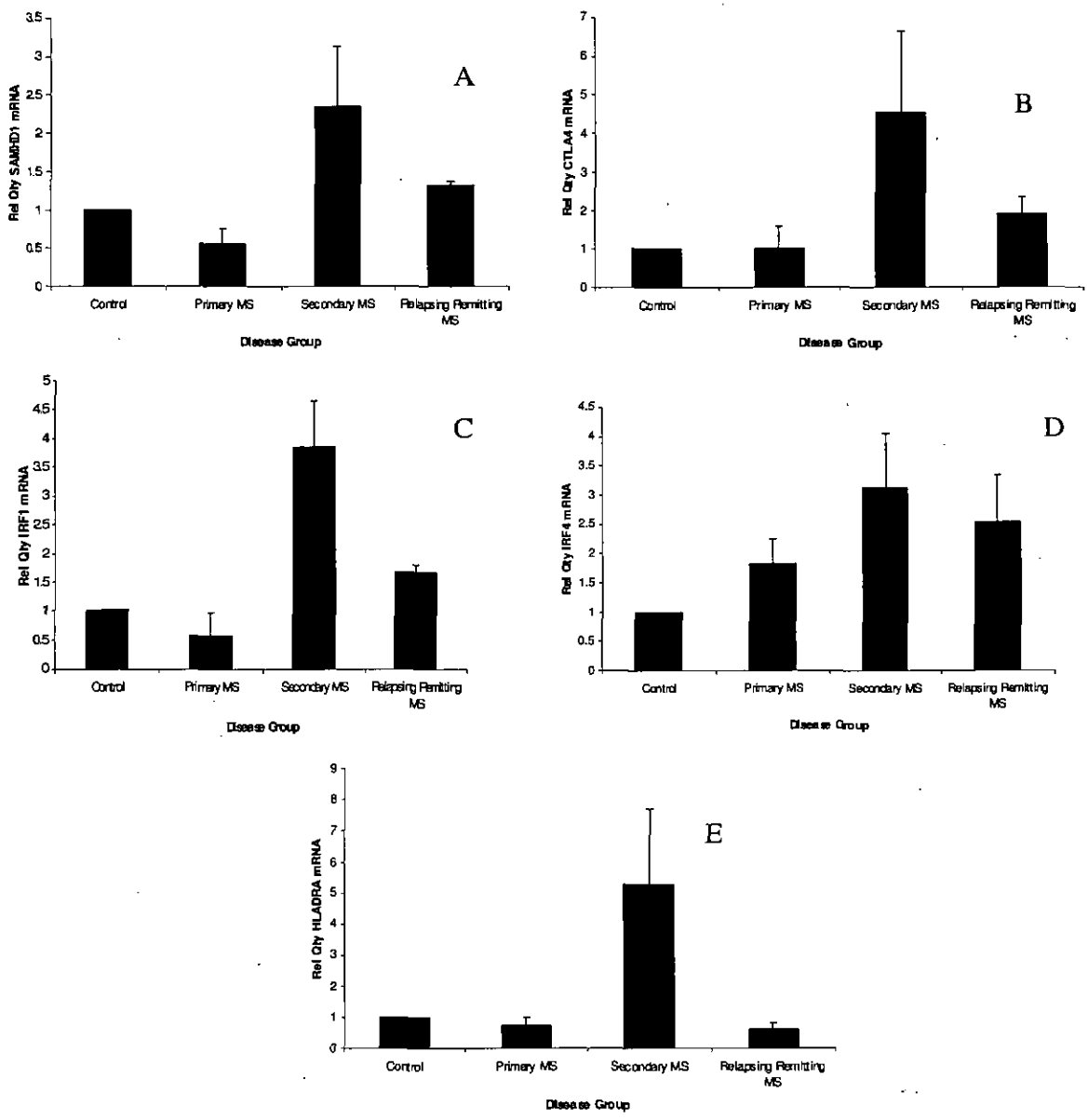


Figure 5.9 Expression of the five selected genes in Multiple Sclerosis

Confirmation of the oligonucleotide microarray identified alterations in A) SAMHD1, B) CTLA4, D) IRF1, E) IRF4, and F) HLADR alpha by quantitative Real time PCR. All expression values were normalised to 18S rRNA to control for equivalence of loading. All measurements were completed in triplicate.

Data are quoted relative to control, which has a value of 1.

5.9.2 Cytotoxic T lymphocyte-associated protein 4

Symbol: CD152, synonymous name: cell differentiation antigen CD152, mapped on chromosome 2q33. CTLA4 is a member of the immunoglobulin superfamily. It is a costimulatory molecule expressed by activated T-lymphocytes. CTLA4 is similar to the T-lymphocyte costimulatory CD28, and both molecules bind to B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells. CTLA4 encodes a protein that transmits an inhibitory signal to T-lymphocytes, whereas CD28 transmits a stimulatory signal. Mutations in the gene are associated with MS [332] also other diseases such as coeliac disease, insulin dependent diabetes mellitus, Graves disease and other autoimmune diseases [333].

Microarray analysis of the patient's blood samples with different MS phenotypes and controls showed upregulation of CTLA4 in the SP MS patient group using GOTERM analysis. To confirm that, Real Time RT-PCR analysis was completed. As can be seen in Figure 5.9 (B), CTLA4 mRNA levels are significantly increased in both SP and RR MS populations, whilst primary MS demonstrates a reduction in measured CTLA4 transcript. A recent study was carried out in Northern Ireland showed an increased rate of carriage of CTLA4 +49 A/G and 3' UTR polymorphism in their PP MS population compared to their RR MS population [334]. This finding is encouraging for us to do further analysis in our patient's samples looking at the type of polymorphism they have.

5.9.3 Interferon regulatory factor 1

Symbol: IRF1, mapped on chromosome 5q31.1. It is the first member of the interferon regulatory transcription factor (IRF) family to be identified, and has been linked to many innate and adaptive responses [335]. It regulates the expression of important immune mediators such as the gene encoding the p35 and p40 subunit of both IL-12 as well as p40 subunit of IL-23. Recently, IRF1 has been shown to be essential in Th-1 differentiation by acting on IL-12rb1, the gene encoding the IL-12 receptor β 1 subunit (IL-12R β1) [336]. Previous data showed that IRF1-deficient mice have impaired Th-1 type immune responses and favoured Th-2 [337] [338]. IRF1 serves as an activator of interferons α and β transcription [339]. IRF2, apparently antagonizes the IRF1 effect by competing for the same cis elements [340].

Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of IRF1 in the SP MS patient group using GOTERM analysis.

To confirm the microarray-identified alterations in IRF1 in MS, real time RT-PCR analysis was completed. As can be seen in Figure 5.9 (C), IRF1 mRNA levels are significantly increased in both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured IRF1 transcript.

5.9.4 Interferon regulatory factor 4

Symbol: IRF4, mapped on chromosome 6p25-p23. It is a lymphoid specific transcription factor expressed on lymphocytes and it is essential for the proliferation and differentiation of B-lymphocytes and proliferation of mitogen-activated T-lymphocytes and therefore plays a crucial role in the function of the immune system. Indeed, IRF4 has been shown to induce IL4 gene transcription [341]. Grossman *et al.* (1996) cloned a new similar human interferon regulatory factor (IRF) named LSIRF for 'lymphocyte-specific IRF' [342]. A regulatory role for IRF4 in the control of IL-6 production by mucosal T-lymphocytes and subsequent T-lymphocyte apoptosis has been demonstrated in a mouse model of colitis [343].

Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of IRF4 in the SP MS patient group using GOTERM analysis.

To confirm the microarray-identified alterations in IRF4 in MS, real time RT-PCR analysis was completed. As can be seen in Figure 5.9 (D), IRF4 mRNA levels are significantly increased in PP, SP and RR MS populations.

5.9.5 Major histocompatibilty complex, classII, DR alpha

Symbol: HLA-DRA1, mapped on chromosome 6p21.3. A class II molecule heterodimer, consisting of α and β chain expressed on antigen presenting cells (APC) and activated T-lymphocytes. It plays a central role in the immune

system by presenting peptides derived from extracellular proteins. Classically, HLA-DR is an immune stimulator but recently it has been shown to play a role as a negative regulator of immune activation expressed on activated lymphocytes [344]. McGuigan *et al*, 2005 carried out a study epidemiological study of MS included seventy-three MS patients from County Donegal and forty-five from County Wexford with 200 control subjects enrolled from each county. This paper examined whether the MS prevalence difference between the two counties is due to population differences in the genetic predisposition to MS. The strong association with the development of MS and the HLA DRB1*1501 and DQB1*0602 phenotypes was confirmed in both patient populations relative to controls. The Donegal sample control population also had a significantly higher carriage rate of the HLA DRB1*1501 – DQB1*0602 haplotype relative to the Wexford sample control [29].

Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of HLA-DRA in the SP MS patient group using GOTERM analysis.

To confirm the microarray-identified alterations in HLA-DRA in MS, real time RT-PCR analysis was completed. As can be seen in Figure 5.9 (E), mRNA levels of HLA-DRA are only significantly increased in SP MS populations.

5.10 Expression of Selected Genes In Vitro

5.10.1 SAM domain and HD domain 1

Figure 5.10 (A) represents the differential gene expression of SAMHD in PBMC from healthy individuals treated with IL2, MBP and IL2 + MBP in comparison to controls (untreated PBMC from the same individual) using real time RT- PCR. Comparing the relative values to the controls, the figure demonstrates no significant change in gene expression profile of SAMHD in PBMC treated with IL2 and IL2 + MBP.

5.10.2 Cytotoxic T-lymphocyte-associated protein 4

Figure 5.10 (B) represents the differential gene expression of CTLA4 in PBMC from healthy individuals treated with IL2, MBP and IL2 + MBP in comparison to controls (untreated PBMC from the same individual) using real time RT-PCR. Comparing the relative values to the controls, the figure demonstrates an increase of 2.15 fold of CTLA4 gene expression in PBMC treated with IL2 (p=0.04) and 1.4 fold for those treated with IL2 + MBP in comparison to controls.

5.10.3 Interferon regulatory factor 1

Figure 5.10 (C) represents the differential gene expression of IRF1 in PBMC from healthy individuals treated with IL2, MBP and IL2 + MBP in comparison to controls (untreated PBMC from the same individual) using real time RT-PCR. Comparing the relative values to the controls, the figure demonstrates no

significant change in gene expression profile of SAMHD in PBMC treated with IL2 and IL2 + MBP.

5.10.4 Interferon regulatory factor 4

Figure 5.10 (D) represents the differential gene expression of IRF4 in PBMC from healthy individuals treated with IL2, MBP and IL2 + MBP in comparison to controls (untreated PBMC from the same individual) using real time RT-PCR. Comparing the relative values to the controls, the figure demonstrates an increase of 1.2 fold of IRF4 gene expression in PBMC treated with IL2 compared to the PBMC treated with IL2 + MBP which shows no significant change.

5.10.5 Major histocompatibility complex, classII, DR alpha-

Figure 5.10 (E) represents the differential gene expression of MHC class II DR α in PBMC from healthy individuals treated with IL2, MBP and IL2 + MBP in comparison to controls (untreated PBMC from the same individual) using real time RT- PCR. Comparing the relative values to the controls, the figure demonstrates a decrease of 0.8 fold of MHC class II DR α gene expression in PBMC treated with IL2 and IL2 + MBP.

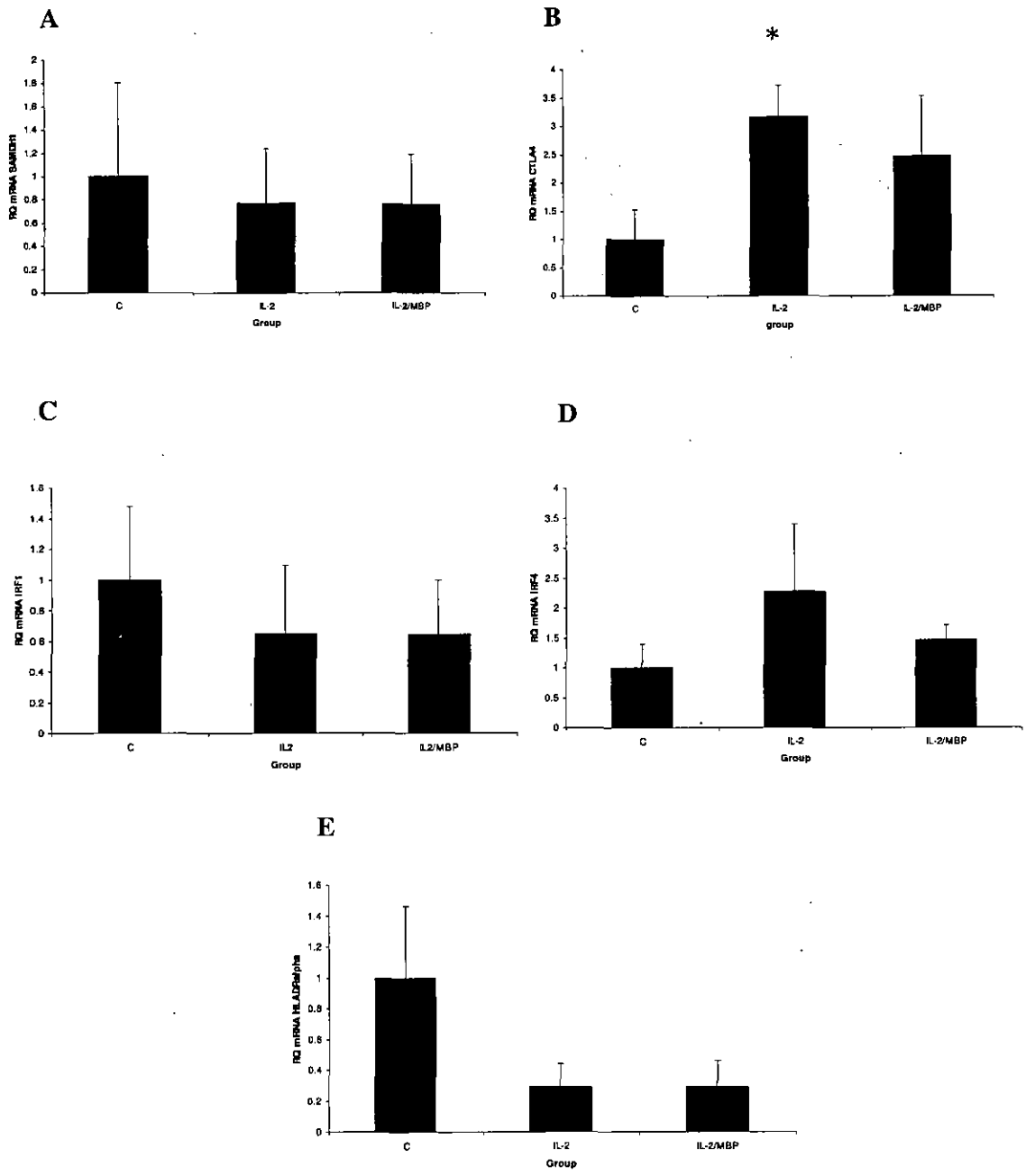


Figure 5.10. Expression of the five genes – *in vitro*

Represents gene expression of A) SAMHD1, B). CTLA4, C). IRF1, D). IRF4, and E). HLADR alpha in PBMC from healthy individual treated with IL2 and IL2 +MBP in comparison to untreated PBMC from the same individual using RT- PCR. *$p<0.05$ versus C

5.11 Discussion

As mentioned previously, MS is a polygenic inflammatory autoimmune disease of the CNS. It may manifest in different clinical forms requiring different management strategies and associated with different outcomes [121]. While there have been major advances in our understanding of MS there remains a gap in very fundamental issues regarding the aetiology and pathogenesis of the disease. Currently, the diagnosis of MS is established through a combination of clinical, laboratory and radiological data [100]. A pathological diagnosis is rarely established in life. In addition, we are still unable to treat MS in a targeted manner, treatment approaches is still empirical in the absence of solid scientific consensus on the specific mechanisms involve in disease development and progression.

The current hypothesis for the development of MS is that T-lymphocytes in genetically susceptible individuals are activated in the peripheral circulation and then migrate to CNS. This understanding is based on alterations in the blood and CSF as well as pathological features in the brain [153] and by analogy with experimental autoimmune encephalomyelitis (EAE) [156].

So far, it has been established that MS is more likely to occur in close relatives than in the general population [255;345]. In spite of extensive studies we still have not been able to identify a causative gene or establish a mode of inheritance. As a consequence, MS is now recognised as a polygenic disease, in which multiple loci could be contributory [255;346].

Over the past decade, researchers have begun to employ new strategies to study complex genetic diseases. One of the most potent strategies employed by researchers is gene expression microarray technology. This technology measures the relative abundance of messenger RNA (mRNA) of thousands of genes in parallel. One of the major advantages of this technique is no prior bias is established in the analysis, given the genome wide nature of the screening process and is therefore an excellent approach to identify novel genes. In 1999, Whitney *et al* used this technique to study gene expression profiling of MS tissue. Since then, multiple studies were carried out on MS lesions as well as peripheral blood of MS patients. In 2003 this approach was utilised by Bomprezzi *et al* to study gene expression of peripheral blood mononuclear cells to identify novel genes that contribute to the susceptibility to MS [329]. Amongst their findings is the reduced transcription of the heat shock protein –70, together with the group of histones and the kinase-2 of the CDC28 complex (CKS2). Of note the study again highlighted the importance of chromosome region 6p21 as it harbours both the HLA complex, the histone cluster and the heat shock protein-70.

In this chapter, we have employed the same microarray technology (Affymatrix U133A plus) in an attempt to define whether disease phenotype specific differences exist in the T-lymphocyte transcriptome relevant to the presenting and pathological features occurring across the spectrum of MS. Identification of disease modifying changes in T-lymphocyte gene expression could help to solve some of the deficits in the management of this most complex disease.

Hence we studied gene expression in T-lymphocytes isolated from patients with different MS clinical phenotypes and those of healthy individuals. Data generated from samples taken from patients with RR MS, SP MS and PP MS were compared to those of healthy individuals. The results showed 178 genes were significantly upregulated and 506 genes were significantly downregulated for the RR MS group, 105 genes were significantly upregulated and 273 genes were significantly downregulated for the SP MS group, and 246 genes were significantly upregulated and 314 genes were significantly downregulated for the PP MS group.

A number of genes were identified to be commonly upregulated in the progressive groups of MS, namely S100 calcium binding protein A8, an IFN-γ regulated gene involved in lymphocyte activation [347]. A second gene worth noting is synaptotagmin-like 3, which promotes chemotactic T-lymphocyte migration [348].

Two genes were identified to be commonly upregulated on all 3 groups of MS in comparison to controls. First gene is phosphodiestrase 4B, cAMP-specific induced in T-lymphocyte activation and plays a role in T-lymphocyte proliferation [349;350]. The second gene is adaptor-related protein complex 3mu2 subunit. This gene is involved in clathrin vesicle formation and internalisation and recycling of receptor complex.

From the genes identified as being upregulated in SP MS, we selected a subset of immune related genes that were likely to be implicated in T-lymphocyte

activation and their expression was further probed: mRNA expression of these genes was confirmed in T-lymphocytes obtained from two sources, human T-lymphocytes from patients with the three clinical phenotypes of MS and compared to healthy controls and also in T-lymphocytes extracted from an *in vitro* model of T-lymphocyte activation (exposed to MBP) and compared to control activated T-lymphocytes (not exposed to MBP). Five genes were selected for the above analysis, namely SAM domain and HD domain 1 (SAMHD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), interferon regulatory factor 1 (IRF1), interferon regulatory factor 4 (IRF4), and major histocompatibility complex, class II, DR alpha (MHC class II DR alpha).

The first gene we identified was SAM domain and HD domain 1 (SAMHD1), aka Dendritic cell- derived IFNγ-induced protein; DCIP. Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) able to induce a primary T-cell response in vivo. This gene had not previously been identified in T-lymphocytes or associated with MS. Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of SAMHD1 in the SP MS patient group. To confirm the microarray identified alterations in SAMHD1 in MS, real time RT-PCR was employed and showed SAMHD1 mRNA levels are significantly increased in both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured SAMHD1 transcript. This may be explained as it has the potential to induce T-lymphocyte responsiveness (including induction of IFN-γ) and hence promotion of a proinflammatory environment. Despite these findings, we did not detect a

significant change in SAMHD1 expression in primed T-lymphocytes obtained from PBMC treated with IL2 and IL2 +MBP.

The second gene we examined was cytotoxic T-lymphocyte-associated protein 4, (CTLA-4), aka: cell differentiation antigen CD152, a member of the immunoglobulin superfamily coded from chromosome 22q33. It is a costimulatory molecule expressed by activated T cells. CTLA4 is similar to the T-lymphocyte costimulatory CD28, and both molecules bind to B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells. CTLA4 encodes a protein that transmits an inhibitory signal to T-lymphocytes by binding preferentially to CD80 (a costimulatory T-lymphocyte receptor –TCR [351], whereas CD28 transmits a stimulatory signal. Loss of function polymorphism of the CTLA-4 gene has been associated with MS [352-354] also other diseases such as coeliac disease, insulin dependent diabetes mellitus, Graves disease and other autoimmune diseases [333]. CTLA-1 gene mutation causes inappropriate and prolonged activation of immune responses. Microarray analysis of the patient's blood samples with different MS phenotypes and controls showed upregulation of CTLA4 in the SP MS patient group and this was confirmed using real time RT-PCR analysis which showed a significant increase in both the SP and RR MS populations, with PP MS demonstrating a reduction in measured CTLA4 transcript. CTLA4 mRNA in T-lymphocytes from PBMC cultures from healthy individuals treated with IL2 and IL2 + MBP was increased 2.15 fold in the IL2 treated group when compared to 1.4 fold in the IL2 + MBP group. Our findings are in agreement with the literature regarding mutations/polymorphisms of CTLA4 being associated with prolonged and inappropriate immune response.

Based on that further analysis of our patients' samples may help in identifying the susceptibility mutation.

The third gene studied was Interferon regulatory factor 1 (IRF1), mapped on chromosome 5q31.1. It is a member of the interferon regulatory transcription factor (IRF) family. It serves as an activator of interferons α and β transcription [339]. Fortunato *et al* demonstrated that genetic variants of IRF1 are associated with MS [355]. Taniguchi et al found that IRF1 is essential for naïve T lymphocytes differentiation into Th1 [336]. Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of IRF1 in the SP MS patient group. To confirm the microarray-identified alterations in IRF1 in MS, real time RT-PCR analysis was employed and showed significant increase in both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured IRF1 transcript. Here, our findings are in agreement with IRF1 being essential for naïve T-lymphocyte differentiation into Th1 (proinflammatory). Despite these findings, we did not detect a significant change in IRF1 expression primed T-lymphocytes obtained from PBMC treated with IL2 and IL2 +MBP.

The fourth gene we looked at is Interferon regulatory factor 4 (IRF4), mapped on chromosome 6p25-p23. It is a lymphoid specific transcription factor expressed on lymphocytes and it is essential for the proliferation and differentiation of B-lymphocytes and proliferation of mitogen- activated T-lymphocytes and therefore plays a crucial role in the function of the immune system. Microarray analysis of the patient's blood samples with different

categories of MS and controls showed up-regulation of IRF4 in the SP MS patient group. To confirm the microarray-identified alterations in IRF1 in MS, real time RT- PCR analysis was employed and showed a significant increase in PP, SP and RR MS populations. In T-lymphocytes from PBMC cultures treated with IL2 and IL2 + MBP no alteration in IRF4 gene expression was noted.

The fifth and final gene we looked at is major histocompatibilty complex, classII, DR alpha (HLA-DRA1). It is mapped on chromosome 6p21.3 and plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed on antigen presenting cells (APC). Microarray analysis of the patient's blood samples with different categories of MS and controls showed upregulation of HLA-DRA only in the SP MS patient group. This was confirmed in real time RT-PCR. MHC class II DR α mRNA expression analysis in T-lymphocytes from cultured PBMC from healthy individuals treated with IL2 and IL2 + MBP showed a decrease of 0.8 fold in MHC class II D α expression in the IL2 and IL2 + MBP groups. This is an interesting finding as HLA DR A1 was only overexpressed in our SP MS patients' samples.

5.12 Conclusion

Over the past decade, cDNA microarray technology has been utilised to study gene expression profile in different diseases. As prior knowledge of candidate genes is not required, it is in particular useful in studying polygenic diseases. The method allows the analysis of large numbers of genes and proteins

simultaneously and opens the opportunities to examine their complex interactions.

Our microarray study produced a large amount of data but due to limited time and resources we were able to further study five genes only. Our findings for CTLA4 and IRF1 are consistent with the knowledge of their association with MS. Other immune associated genes like SAMDH1 and IRF4, not previously known to be associated with MS, showed an association in our patients' samples. One interesting finding is the overexpression of HLA-DRA1 only in our secondary progressive patients' samples. Of specific interest is further characterization of the role of S100A8 in MS T-lymphocytes activation as this was the number I upregulated gene in the progressive disease phenotypes.

Chapter 6

Characterizing the molecular genetics of Multiple Sclerosis in unique Irish family

6.1 Aim

In order to improve our understanding of the molecular pathology of MS, we aim to identify genetic linkage to one or more genes in this family.

6.2 Hypothesis

We hypothesise that studying a family with high prevalence of MS may be crucial in identifying some genes that influence MS.

6.3 Objectives

We believe characterisation of the patterns of inheritance and identification of linkage in this family will improve our understanding of genetics underpinning this devastating disease. To genotype an Irish family with high prevalence of MS using Affymetrix 250K Nsp SNP chip. Linkage analysis and significance by simulations were used to identify genomic regions of interest.

6.4 Background and rationale

Although most cases of MS are sporadic, first-degree relatives are at greater risk of developing this disease [206;210;217]. The aetiology of MS involves a complex interplay of genetic and environmental factors with the balance between the two differing between sporadic and familial cases of MS. In family-

based studies of MS genetic susceptibility appears to be the more important influence in the development of MS with multiple genes involved each exerting modest effects. To date, family-based genetic studies in MS have identified associations between MS and polymorphic variants within the major histocompatibility complex (MHC) as well as demonstrating an autosomal dominant pattern of inheritance in certain families, but have not identified a disease-causing gene [122].

In recent time there have been major advances in the field of molecular genetics; the completion of the human genome and HapMap projects combined with high-resolution whole-genome single nucleotide polymorphism (SNP) screening techniques and improvements in statistical methods for data analysis has facilitated the detailed study of specific genes/narrow genomic regions with the aim of identifying genetic variants associated with common diseases (www.hapmap.org). In MS a recently published genome wide association study was able to implicate single nucleotide polymorphisms of the interleukin-2 receptor alpha (IL2RA), interleukin-7 receptor alpha (IL7RA) and those of the HLA locus as risk factors for developing MS. However, our understanding of the genetic susceptibility and inheritance of MS remains far from complete [356].

Linkage is the tendency for genes and other genetic markers to be inherited together because of their close proximity to each other on the same chromosome. DNA segments that lie near each other on the same chromosome tend to be inherited together therefore markers are often used as tools for detecting the inheritance pattern of unidentified genes. LOD score is a statistical

test used for linkage analysis in human population. Developed by Newton E. Morton, it is a statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore likely to be inherited together. A LOD score of 3.0 or more is considered as evidence for linkage, on the other hand a LOD score of –2.0, excludes linkage.

As part of our research project: "*The Identification of The Molecular Pathways and molecular determinants of Multiple Sclerosis*" we identified an Irish family in which there is a very high prevalence of multiple sclerosis: this family comprises of two unaffected parents and eleven children of which four siblings (two males and two females) are affected with MS. Both parents and grandparents of affected patients were all born in Ireland. The medical history of the parents and unaffected siblings is unremarkable for other neurological or auto-immune diseases (e.g. Systemic Lupus Erythematosis), and history of consanguinity. Next all affected and unaffected members of the pedigree had magnetic resonance imaging (MRI) of brain to define the affected status of all family members according to McDonald's criteria [100].

6.5 Results and discussion

Both parents and grandparents of affected patients were all born in Ireland. The medical history of the parents and unaffected siblings was unremarkable for other neurological or auto-immune diseases. Furthermore, there was no history of neurological disease documented in the extended family of nine paternal siblings and four maternal siblings. Although previous studies in pedigrees have reported an autosomal dominant pattern of inheritance, in this unique Irish

pedigree MS appears to behave as an autosomal recessive/ autosomal dominant with incomplete penetrance trait due to absence of disease in the first generation (parents). Four clinically affected members had MRI brain imaging consistent with MS. All four met the McDonald criteria of MS. HLA genotype did not explain affected status. The mother was a homozygote for HLA-DR15 and HLA-DQ6. The father did not have this haplotype, but all family members (children) had one haplotype of HLA-DR15 and HLADQ6, received from their mother (Table 6.1). In addition, the chromosomal analysis of one affected individual was normal. One affected individual was randomly chosen as a representative of having the same karyotype as all the affecteds.

Estimation of the power and type I error of this pedigree to detect linkage using a traditional parametric approach, under two genetic models for the disease locus was performed using SIMLINK [357]. A recessive disease model was specified with a completely penetrant recessively acting disease allele with population frequency of 0.50, linked to a marker allele with frequency of 0.2. Simulated genotypes were generated 10,000 times and the mean and maximum LOD scores with the marker locus, were calculated as mean = 0.635 and maximum (theta = 0) = 2.521.The probability of observing a LOD score of 0.5 at theta=0 was 0.496. Under the null hypothesis (no linked disease locus) the mean LOD score was -0.935, with theta set = 0.01. The probability of observing a LOD score of -2 (traditional criterion for the rejection of linkage) was 0.770 in the absence of a linked locus (theta = 0.01).

A dominant genetic model was also simulated, with incomplete penetrance =

0.60 in the presence of one or two disease alleles, and a population disease allele frequency of 0.005. The mean LOD score was 0.186, and the maximum 0.887 with a completely linked marker locus (theta=0). The power to detect the disease locus with a LOD score of 0.5 at theta=0 was 0.274. In the absence of a disease locus linked to the marker, the mean LOD score observed was -0.129, at theta = 0.01. The probability of observing a LOD score of -2 in the absence of a linked disease locus was approximately 1.

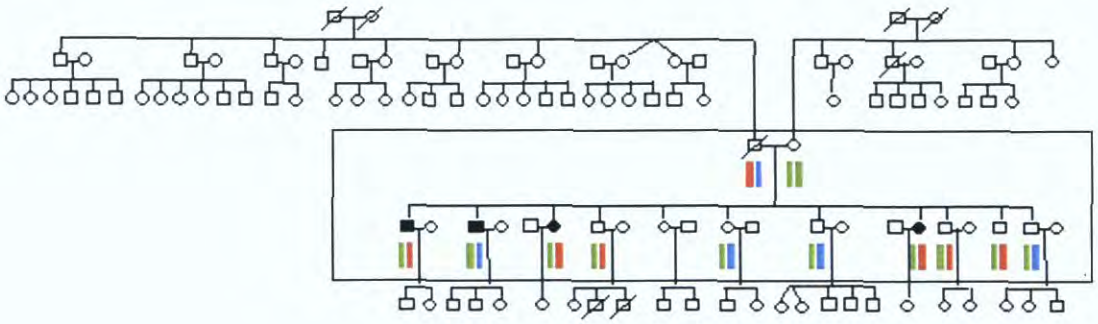
These simulations demonstrate that this family has zero power to detect a true linkage at a significant LOD score (LOD=3), the principal problem being a lack of informative meioses. Power to detect a recessive locus was better than that to detect a dominant locus with incomplete penetrance. However, it is relatively likely that under either model, the maximal LOD score obtainable will be observed somewhere in the genome, either by chance, or due to a true linkage signal.

Linkage analysis showed seven chromosomal regions with maximal non-parametric LOD score: chromosomes 5q, 6q, 7q, 11p, 12q, 13q and 20q (Table 6.2; Figure 2). These did not attain statistical significance at a genome-wide level, from 200 simulations under the null hypothesis of no linkage. The results of the locus-counting analysis (table 6.3) showed that we obtained an excess of linkage peaks, especially at lower LOD score thresholds, and in the non-parametric and recessive analyses rather than in that under partial dominance. However, this analysis cannot indicate which of the peaks may be true positives.

None of these regions appear to have shown persuasive evidence for linkage in previous studies, therefore if they are true findings they may represent loci specific to this pedigree, or the Irish population. However, a region on chromosome 6q showed a suggestive significance level in a recent meta-analysis of genome-wide linkage analysis [358]. Several of these regions contain interleukin, tumour necrosis factor (TNF), and integrin related genes that are of potential interest, and will be followed up (Table 6.3).

6.6 Conclusion

The inheritance of MS in this unique Irish family is not due to HLA status and therefore offered an opportunity to identify Mendelian genes involved in MS genetics. Power calculations indicated that there was a low probability of detecting a locus at a statistically significant level of 0.05, and in fact no genome-wide significance was obtained. While this makes it difficult to draw a definitive conclusion about the highest linkage peaks, there are clearly regions of the genome that are more highly shared by the four affected siblings present in this pedigree than the unaffected. It was of interest that both HLA typing, and the linkage analysis failed to implicate the HLA locus on chromosome 6p. While it remains possible that other haplotypes at this locus are risk factors in this family, which the linkage analysis was underpowered to detect, it is also possible that independent, distinct genetic loci are responsible for the high incidence of MS in this pedigree. A potential caveat here is that the possibility remains that further siblings may become affected in the future, in which case this analysis must be updated.



DRB1*0401/0413- DQB1*0302/0305

DRB1*15-DQB1*0602/0611

DRB1*1301/1302-
DQB1*0609/0618

Figure 6.1. Schematic demonstration of the unique Irish pedigree also demonstrating their HLA status.
NB. Only individuals included in the box were studied.

Patient	Sex	Age (yrs)	years since Dx	MS category	EDSS	MRI Brain	LP	DMA	PMHX	Other Medication
C3	M	48	10	RR	2	+	+	Interferon β 1a	Nil	none
C4	M	47	27	SP	6.5	+	+	Interferon β 1b	IVDA – Hep C	Baclofen Methadone
C5	F	45	4	RR	0	+	+	Galtirmer acetate	Nil	none
C8	F	40	1	RR	0	+	+	Galtirmer acetate	Migraine	none

Table 6.1. Affected patients data summary

Chromosome	Genetic position (Marshfield cM)	SNP boundaries
5q35.1 - qter	186 – 196	rs2052454 - rs7735361
6q22.2 - q24	126 – 141	rs6938035 – rs2328162
7q36.1 – q36.3	170 – 175	rs758315 – rs10261578
11p14 - p11	36 – 55	rs10835649 – rs835835
11q14.1	89	rs2512390 – rs10898776
12q13.11 - q15	64 – 80	rs3741630 – rs2305641
13q33.3 - q34	95 – 113	rs9301199 – rs7400722
20q11.2 – q13.2	51 – 74	rs1016118 - rs2426204

Table 6.2. Summary of maximal LOD score chromosomal peak positions, and marker boundaries.

Symbol	Cytogenetic location	Gene Description
IFNGR1	6q23-q24	interferon gamma receptor 1
CAT	11p13	catalase
CD59	11p13	CD59 molecule, complement regulatory protein
SLC1A2	11p13-p12	solute carrier family 1 (glial high affinity glutamate transporter), member 2
SCN8A	12q13	sodium channel, voltage gated, type VIII, alpha
TAC3	12q13-q21	tachykinin 3 (neuromedin K, neurokinin beta)
LRP1	12q13-q14	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)
CYP27B1	12q13.1-q13.3	cytochrome P450, family 27, subfamily B, polypeptide 1
IL23A	12q13.2	interleukin 23, alpha subunit p19
IFNG	12q14	interferon, gamma
MMP19	12q14	matrix metallopeptidase 19
TNFSF13B	13q32-34	tumor necrosis factor (ligand) superfamily, member 13b
MMP9	20q11.2-q13.1	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
CD40	20q12-q13.2	CD40 molecule, TNF receptor superfamily member 5

Table 6.3. Overlap of genes lying under our top linkage peaks (table 6.2) with a GeneCards search for keywords "Multiple Sclerosis".

Figure 6.2. Summary of chromosomes showing maximal nonparametric LOD scores (right-hand axis, dark blue line); also shown recessive (light *blue*) and *dominant* (red) parametric linkage scores (left-hand axis).

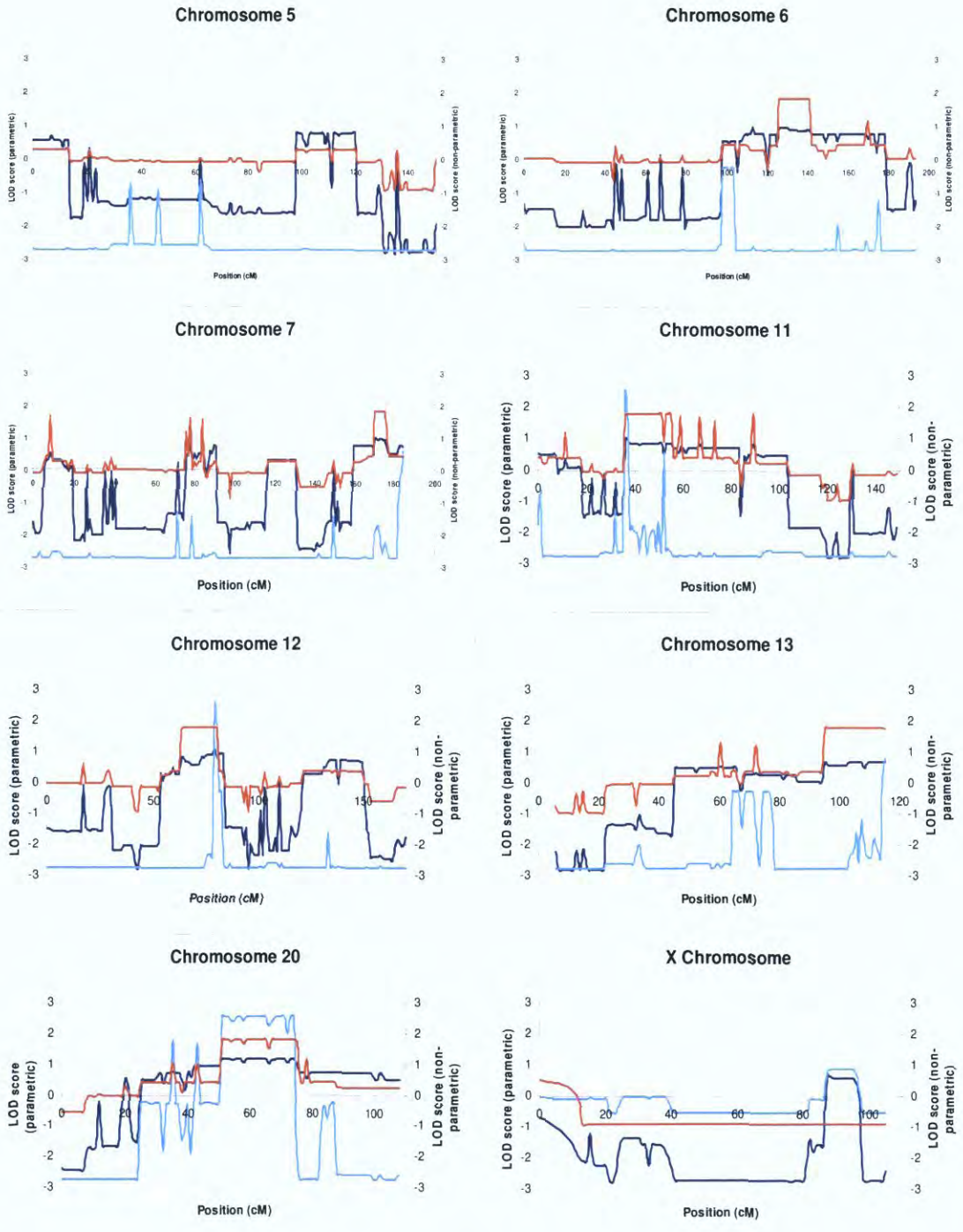


Figure 6.3 A. Summary of other chromosomes (right-hand axis, dark blue line); also shown recessive (light blue) and dominant (red) parametric linkage scores (left-hand axis).

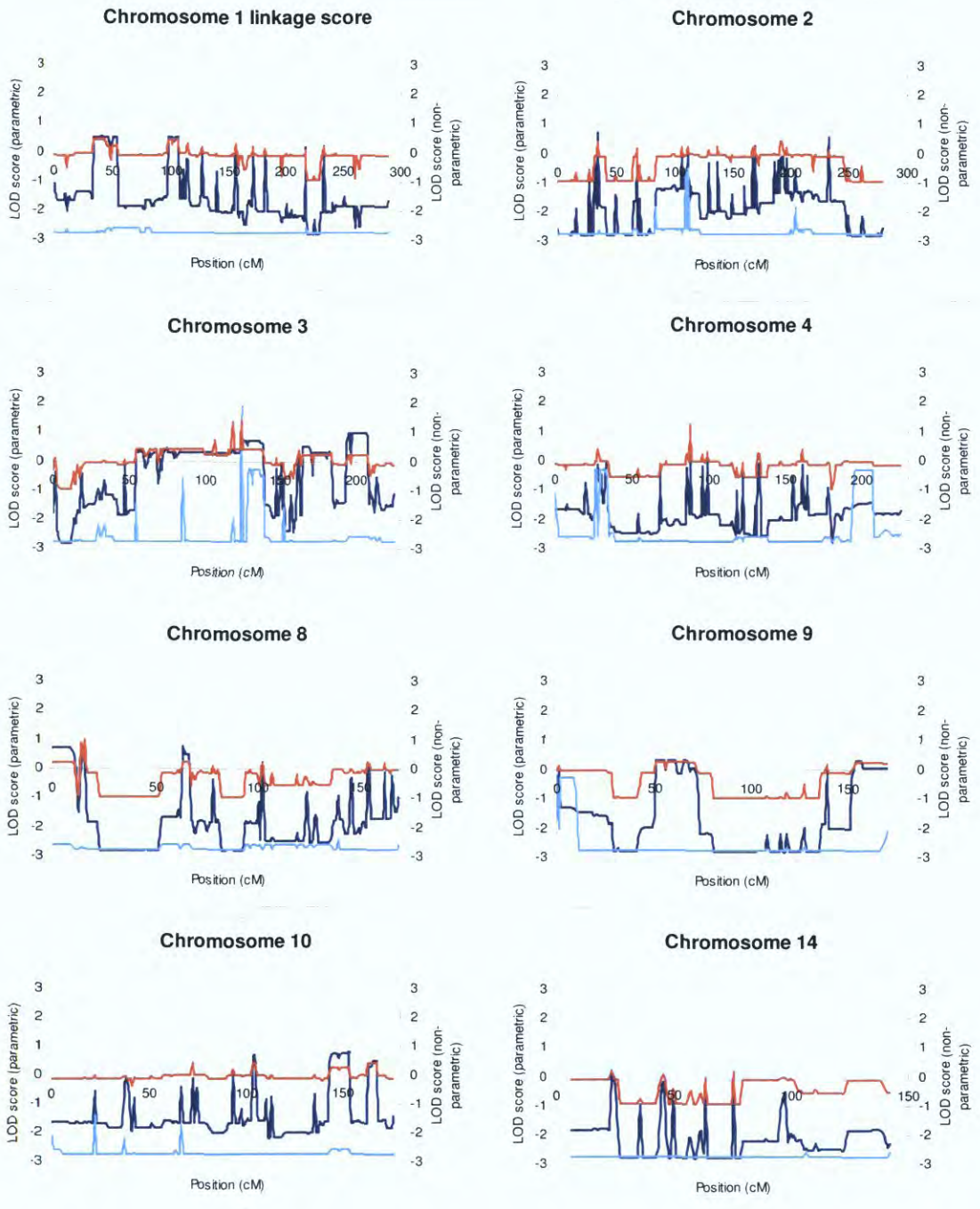
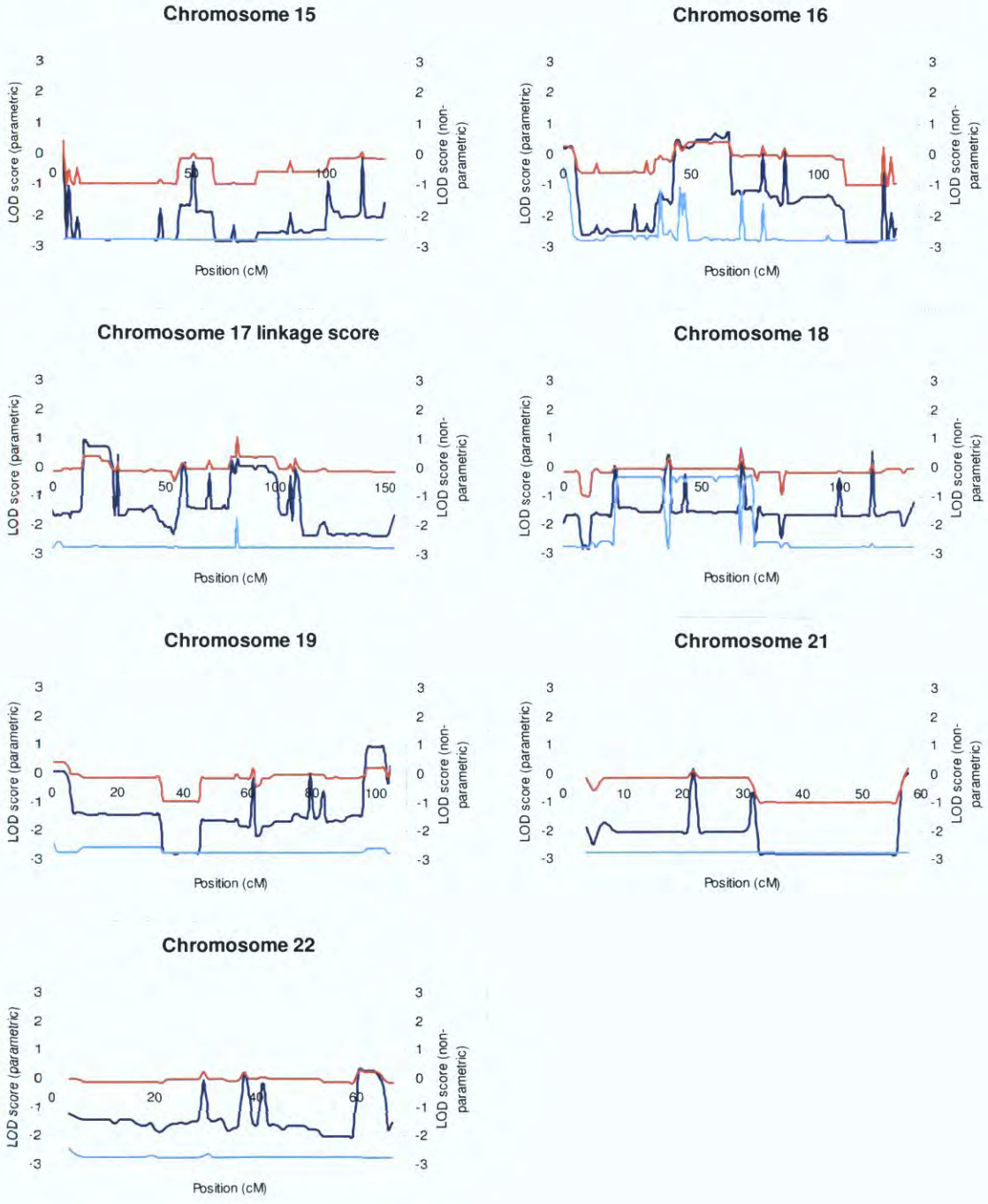


Figure 6.3 B. Summary of other chromosomes (right-hand axis, dark blue line); also shown recessive (light blue) and dominant (red) parametric linkage scores (left-hand axis).



Summary and Final Conclusion

While significant advances have been made in understanding the pathophysiological basis for MS symptomology and its natural history in terms of progression, a definitive answer on the origin of the disease is still debated. As with other inflammatory autoimmune diseases, strong arguments exist pointing towards the importance of genetic predisposition in disease susceptibility. Aside from the long established heritability of MS, regarding the precise the molecular genetics of this effect, the only concrete evidence of a genetic influence to date centres on the role of HLA class II haplotypes. Various studies abound on the role of other genes as risk modifiers in MS.

However, another aspect of MS in which molecular genetics may play a role is in determining the disease phenotype observed in an affected patient. As MS is a T-lymphocyte mediated disease, this thesis aimed to explore the logical hypothesis that changes in T-lymphocyte gene expression profiles, may represent an important determinant of disease phenotype. The potential clinical relevance of such an effect cannot be underestimated. Firstly, it would allow for a more rational design of phenotype specific treatment regimens and secondly also facilitate a more defined method of predicting disease phenotype at the time of diagnosis. Related to this, and of relevance to any future advances in the area, the fact that T-lymphocytes can be obtained from peripheral blood samples, permitted an experimental design that precluded the requirement for

cerebrospinal fluid sampling, thereby limiting the discomfort and inconvenience experienced by the participating patients.

The first approach undertaken in this thesis was based on the use of the world wide web available (*in silico*) technology, digital differential display (DDD), in order to identify a cohort of genes found to be expressed in MS brain and whose function might be implicated in T-lymphocytes activation. As access to brain tissue is limited, the use of DDD as an online repository of gene expression profiles has made it more simple to obtain and compare MS brain tissue profiles with those of controls.

In total 31 EST's were identified using DDD, of those four genes, namely ANXA1, ZHX1, PRNP and APOE were selected to study their expression in T-lymphocytes obtained from patients with different MS clinical phenotypes and compared them to those of healthy controls. Looking at the expression of those genes in peripheral T-lymphocytes we were able to demonstrate that both ZHX1 and PRNP had increased expression in the RR MS and SP MS phenotypes. The gene expression of ANXA1 was increased in the SP MS phenotype, whereas APOE gene expression was decreased in the PP MS phenotype. The results of this study showed that the rationale employed for linking data from available online sources to hypothesis testing was effective to some extent. In this case we were able to show common phenotype-specific patterns of gene expression, albeit based on a small sample size in each cohort.

The second approach was based on examining global gene expression in T-lymphocytes obtained from patients with different MS clinical phenotypes and controls using microarray technology (Affymetrix U133A plus 2.0) in an attempt to define whether disease phenotype specific differences exist in the T-lymphocyte transcriptome. The ultimate aim of such a study is to forward a rationale for the hypothesis that patients might be able to be stratified into disease subgroups, according to a particular T-lymphocyte profile. This might allow for more focused treatment approaches in a manner analogous to the current major research effort to identify drug response predicting biomarkers in cancer. Implicit in the ability to identify disease specific treatments would be the ability to establish a mechanistic link between T-lymphocyte gene expression profiles, phenotype-specific pathophysiological features and ultimately patterns of clinical disease behaviour.

One of the major advantages of this technique is no prior bias is established in the analysis, given the genome wide nature of the screening process and is therefore an excellent approach to identify novel genes. The results showed 178 genes were significantly upregulated and 506 genes were significantly downregulated for the RR MS group, 105 genes were significantly upregulated and 273 genes were significantly downregulated for the SP MS group, and 246 genes were significantly upregulated and 314 genes were significantly downregulated for the PP MS group.

A number of genes were identified to be commonly upregulated in the progressive groups of MS, namely S100 calcium binding protein A8, an IFN-γ

regulated gene involved in lymphocyte activation. A second gene worth noting is synaptotagmin-like 3, which promotes chemotactic T-lymphocyte migration. Two genes were identified to be commonly upregulated on all 3 groups of MS in comparison to controls. Phosphodiesterase 4B, a cAMP-specific protein induced in T-lymphocyte activation and playing a role in T-lymphocyte proliferation. Adaptor-related protein complex 3mu2 subunit.-the gene product of which is involved in clathrin vesicle formation and internalisation and recycling of receptor complexes-was also increased in all three groups.

Subsequently we sought to validate by real-time RT-PCR, the expression of selected immune-related genes that were altered on the MS microarrays and thought to represent likely modulators of T-lymphocyte activation, namely SAMHD1, CTLA4, IFN1, IFN4 and HLA DR alpha in T-lymphocytes from MS patients with different clinical phenotypes. We further examined the expression of these genes in T-lymphocytes from normal donors activated *in vitro* with MBP and IL-2.

The first gene we identified was SAMHD1, which showed a significant increase in expression in T-lymphocytes obtained from both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured SAMHD1 transcript. No previous studies have demonstrated SAMDH1 to be associated with MS, but SAMDH1 is known to have the potential to induce T-lymphocyte responsiveness (including induction of IFN-γ) and hence promotion of a proinflammatory environment as seen in both RRMS and SPMS phenotypes.

Despite these findings, the *in vitro* experiment work failed to show that SAMDH1 expression was induced in autoreactive T-lymphocyte cultures.

CTLA4 showed a significant increase in its expression in T-lymphocytes obtained from both the SP and RR MS populations, with PP MS demonstrating a reduction in measured CTLA4 transcript. CTLA4 expression was increased following IL-2 treatment of cultured control donor T-lymphocytes, implicating it in T-lymphocyte proliferation. From the literature, mutations in the CTLA4 gene are known to be associated with MS. Thus our findings are in agreement with the literature regarding CTLA4 being associated with prolonged and inappropriate immune response seen in both RR and SP MS phenotypes. Based on this, further analysis of our patients' samples may help in identifying the susceptibility mutation.

IRF1 showed a significant increase in its expression in T-lymphocyte in both SP and RR MS populations, whilst PP MS demonstrates a reduction in measured IRF1 transcript. IRF1 serves as an activator of interferon α and β transcription and is essential for naïve T-lymphocyte differentiation into Th1 (proinflammatory) therefore it is not surprising to find it overexpressed in patient samples in the RR and SP MS. Despite these findings, the *in vitro* experiment work did not show a significant difference in IRF1 expression.

IRF4 showed a significant increase in the T-lymphocytes obtained from PP, SP and RR MS populations. It is a lymphoid specific transcription factor expressed on lymphocytes and it is essential for the proliferation and differentiation of B-

lymphocytes and proliferation of mitogen- activated T-lymphocytes and therefore plays a crucial role in the function of the immune system. Again our *in vitro* experiment did not provide us with any further information.

The fifth and final gene studied was HLA-DRA1. This was only over-expressed in our SP MS patients' samples. Strikingly, an 80% decrease in expression was observed in the IL2 and IL2 + MBP treated groups of control T-lymphocytes, indicating a negative role for IL2 and perhaps proliferation in HLA-DRA1 expression.

Examining the functional role of the microarray identified genes was a major limitation in this thesis. The association of gene expression and an MS relevant functionality was attempted to be studied using a model that creates clonal expansion of MBP specific T-lymphocytes. As the *in vitro* environment in which T-lymphocytes expansion occurs in this model is markedly different from that in which autoimmunity develops *in vivo,* this may have contributed to the relative lack of coherence between disease derived and *in vitro* activated *T*-lymphocytes. In order to match like with like in an MBP model, it may have proven more ideal to have used an *in vivo* MBP based model of experimental encephalopathy from which circulating T-lymphocytes could have been extracted.

In the final section of work in this thesis examined the inheritance pattern of MS in an Irish family with four affected siblings. As the HLA status clearly did not explain the pattern of inheritance in this family and therefore offered an

opportunity to identify Mendelian inherited genes involved in MS genetics. Linkage analysis showed seven chromosomal regions with maximal non-parametric LOD score: chromosomes 5q, 6q, 7q, 11p, 12q, 13q and 20q. These did not attain statistical significance at a genome-wide level, from 200 simulations under the null hypothesis of no linkage and indeed none of these regions appear to have shown persuasive evidence for linkage in previous studies. Overall, the study was underpowered as only two generations were examined, this makes it difficult to draw a definitive conclusion about the highest linkage peaks. However, there are clearly regions of the genome that are more highly shared by the four affected siblings present in this pedigree than the unaffected. Several of these regions contain interleukin, tumour necrosis factor (TNF), and integrin related genes that are of potential interest, and will be followed up.

In summary, this thesis has principally explored the potential of examining gene expression profiles in T-lymphocytes as a way of delineating MS phenotypes. T-lymphocytes drive MS development and progression and are therefore perhaps the most interesting cell type in which to study whether a gene expression profile phenotype-relationship exists in MS.

The largest and most useful set of data generated was derived from the microarray study in which a large number of changes in gene expression were observed in MS T-lymphocytes. Gene expression profiles demonstrated both consistent similar changes in MS *per se,* versus control but also was able to identify putative "phenotype-specific" profiles. Examination of characteristic

phenotype specific gene expression profiles in a much larger cohort is both warranted and necessary before strong conclusions can be drawn.

However, in the event of confidently making such an association, the generation of a faithful and accurate replication of MS lesions in an animal model would be of particular use in terms of examining the impact of these disease specific profiles. For example, given the ease with which genetic knockdown of genes can be established in mouse lines, the effect of loss or gain of particular genes on lesion pathology could offer interesting insights into phenotype specific aspects of pathology. (1,840 words)

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